

G protein-coupled receptor kinase 2 is a Rho-dependent scaffold protein for the ERK MAPK cascade

James Daniel Robinson

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Medical Research Council Laboratory for

Molecular and Cellular Biology

University College London



Declaration

I, James Daniel Robinson confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed..... Date.....

Abstract

The G protein-coupled receptor kinases (GRKs) are best known for their role in phosphorylating and desensitising G protein-coupled receptors (GPCRs). The GRKs can also regulate signalling downstream of other families of receptors and are now known to have a number of non-receptor substrates and binding partners. Here I identify RhoA_{GTP}, Raf1 and ERK2 as novel binding partners of GRK2 and report a previously unsuspected function for this kinase. GRK2 acts as a RhoA-activated scaffold protein for the ERK MAP kinase cascade downstream of the epidermal growth factor (EGF) receptor. The ability of GRK2 to bind to Raf1, MEK1 and ERK2 is dependent on RhoA_{GTP} binding to the catalytic domain of the kinase, however, while RhoA_{GTP} binding is common to all of the ubiquitously expressed GRKs, the ability to act as a RhoA-regulated Raf/MEK/ERK scaffold is specific to GRK2. GRK2 over-expression in HEK-293 cells potentiates EGF-induced ERK activation in a Rho-dependent fashion. Conversely, depleting GRK2 expression by RNAi reveals that GRK2 is required for EGF-induced thymidine incorporation in vascular smooth muscle cells (VSMCs). Rho-dependent ERK MAP kinase scaffolding by GRK2 may therefore have an important role in the vasculature, where increased levels of GRK2 and RhoA have been associated with hypertension.

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Abbreviations used in this thesis

Akt: AkT8 virus oncogene cellular homologue

ANG_{II}: Angiotensin II

APC: Adenomatous polyposis coil protein

Arf6: ADP ribosylation factor 6

ASK1: Apoptosis signal regulating kinase 1

ATP: Adenosine triphosphate

β_1 AR: β_1 adrenergic receptor

β_2 AR: β_2 adrenergic receptor

β ARK_{ct}: β -adrenergic receptor kinase carboxyl-terminal region

BSA: Bovine serum albumin

CAT: Catalytic domain

Cdc42: Cell division control protein homologue 42

CDK2: Cyclin-dependent kinase 2

CK2: Casein kinase 2

CNK1: Connector enhancer of KSR-1

CT: Carboxyl-terminal region

DAG: Diacylglycerol

DGK θ : Diacylglycerol kinase θ

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulphoxide

DREAM: Downstream regulatory element antagonist modulator

DTE: Dithiothreitol

ECL: Enhanced chemiluminescence

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ENaC: Epithelial sodium channel

ERK: Extracellular signal-regulated kinase

ERM: Ezrin/radixin/moesin

eNOS: Endothelial nitric oxide synthase

FCS: Foetal calf serum

GAP: GTPase-activating protein

GDI: Guanine nucleotide dissociation inhibitor

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GFP: Green fluorescent protein

GIT1: GRK interacting protein 1

GLUT4: Glucose transporter 4

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

GST: Glutathione S-transferase

GTP: Guanosine triphosphate

HEK-293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

Hep2: Human epithelial cell line 2

Hsp90: Heat shock protein 90

IB: Immunoblot

IGF1R: Insulin-like growth factor 1 receptor

I κ B: Inhibitor of κ B

IKK: Inhibitor of κ B kinase

IP: Immunoprecipitation

IPTG: Isopropyl- β -D-thio-galactoside

IP₃: Inositol 1,4,5-triphosphate

IRS-1: Insulin receptor substrate 1

IVT: *In vitro* translated

JNK: c-Jun N-terminal kinase

KSR: Kinase suppressor of Ras

LARG: Leukemia-associated Rho GEF

LB: Luria broth

LPA: Lysophosphatidic acid

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MAPKK: Mitogen-activated protein kinase kinase

MAPKKK: Mitogen-activated protein kinase kinase kinase

MDCK: Madin-Darby canine kidney

mDia: Mammalian diaphanous homologue 1

Mdm2: Murine double minute oncogene 2

MEF: Mouse embryonic fibroblast

MEK: MAPK/ERK kinase

MKK: MAPK kinase

Morg-1: MAPK organiser 1

MP-1: MEK partner-1

M1MR: Muscarinic type I receptor

M2MR: Muscarinic type II receptor

M3MR: Muscarinic type III receptor

Nedd4: Neural precursor cell expressed developmentally down-regulated 4

NFκB: Nuclear factor kappa enhancer of activated B cells

NHERF: Sodium/hydrogen exchanger regulatory factor

NO: Nitric oxide

NT: Amino-terminal region

PAK: p21-activated kinase

PBS: Phosphate-buffered saline

PDEγ: Phosphodiesterase γ

PDGF: Platelet-derived growth factor

PDGFR: Platelet-derived growth factor receptor

PEA-15: Phosphoprotein enhanced in adipocytes 15

PH: Pleckstrin homology

PhLP: Phosducin-like protein

Pin1: Prolyl-isomerase 1

PIP₂: Phosphatidylinositol 4,5-bisphosphate

PI3K: Phosphoinositide 3-kinase

PKA: cAMP-dependent protein kinase

PKC: Protein kinase C

PKN: Protein kinase N

PLCβ: Phospholipase C-β

PTD: Protein translocation domain

PTH1R: Parathyroid hormone receptor type 1

Rac: Ras-related C3 *botulinum* substrate

Raf1: Rapidly accelerated fibrosarcoma associated gene 1

RalA: Ras-related protein A

Ras: Rat sarcoma associated gene

RBD: Rho-binding domain

RGS: Regulator of G protein signalling

RH: RGS homology

RKIP: Raf kinase inhibitor protein

ROCK: Rho-associated kinase

ROS: Rod outer segments

RSK-2: Ribosomal S6 kinase 2

RT: Room temperature

RTK: Receptor tyrosine kinase

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

siRNA: Small inhibitory ribonucleic acid

SOC: Super-optimal broth with catabolite repression

Src: Sarcoma virus homologue

S1P: Sphingosine-1-phosphate

SRF: Serum response factor

TBS: Tris-buffered saline

TCA: Trichloroacetic acid

TEMED: Tetramethylethylenediamine

TGF- β : Transforming growth factor β

TNF- α : Tumour necrosis factor α

TPL2: Tumour progression locus 2

TTBS: Tween/tris-buffered saline

VPAC2: Vasoactive intestinal peptide receptor 2

VSM: Vascular smooth muscle

VSMC: Vascular smooth muscle cell

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1. Introduction

1.1. G protein-coupled receptor kinases (GRKs).

The G protein-coupled receptor kinases (GRKs) are a family of seven serine/threonine protein kinases with important and varied roles in regulating cellular signalling, including the phosphorylation and desensitisation of agonist occupied G protein-coupled receptors (GPCRs) (Pitcher et al., 1998a; Premont et al., 1995). They are separated into 3 subfamilies based on sequence comparisons. Both members of the GRK1 subfamily, GRKs 1 and 7, are expressed in the retina and GRK1 is additionally expressed in the pineal gland. The GRK2 subfamily, consisting of GRKs 2 and 3, and GRKs 5 and 6 of the GRK4 subfamily are widely expressed. GRK4 is found at significant levels only in the testis, kidney and cerebellum. All family members share a highly conserved central catalytic domain that is flanked by a more variable amino-terminal region and a poorly conserved, variable length carboxyl-terminal region (Premont and Gainetdinov, 2007; Ribas et al., 2007).

The amino-terminal regions of the GRKs all contain a regulator of G protein signalling (RGS) homology (RH) domain. The RGS domain is a conserved region of approximately 115 amino acids that acts as a GTPase activating protein (GAP) for various $G\alpha$ subunits of heterotrimeric G proteins. The RH domain of GRK2 is known to bind to activated members of the Gq (G_q , G_{11} and G_{14} but not G_{16}) family of heterotrimeric G protein subunits but, unlike the RGS proteins, it does not act as a GAP. Binding partners for the other GRK RH domains remain unidentified (Day et al., 2004). The less conserved carboxyl-terminal regions of the GRKs contain lipid

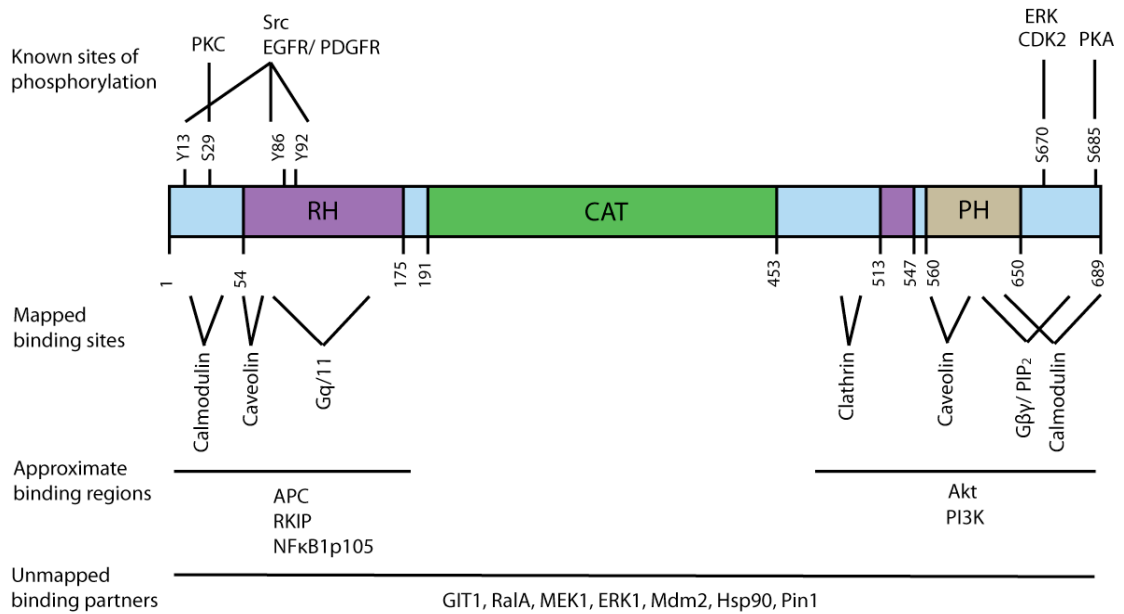
and protein binding sites for constitutive or agonist-dependent membrane targeting as well as sites for regulatory phosphorylation and lipid modification (Pitcher et al., 1998a). GRK2 and GRK3 contain a pleckstrin homology (PH) domain within their carboxyl-terminal regions (amino acids 560-650 in GRK2) that binds co-ordinately to phosphatidylinositol-4,5-bisphosphate (PIP₂) and membrane-localised Gβγ subunits of heterotrimeric G proteins. The PH domain can thus target GRK2 to the plasma membrane in an agonist-dependent manner for receptors that activate heterotrimeric G proteins (Pitcher et al., 1992; Pitcher et al., 1995). In contrast, members of the GRK4 subfamily do not have PH domains but use direct PIP₂ binding and/or palmitoylation for targeting to the membrane.

1.1.1. Structure of GRK2.

Figure 1.1A shows a schematic of the secondary structure of GRK2. The location of the PH domain as well as the catalytic domain and the Gq_{GTP}-binding RH domain of GRK2 are labeled. Known non-receptor binding partners for GRK2 are shown together with their binding sites in the cases where they have been mapped. Also labeled are the known sites of phosphorylation of GRK2 by other protein kinases. The functions of the phosphorylation sites and of the protein-protein interactions shown in Figure 1.1A will be discussed in sections 1.1.3 and 1.1.6. Crystal structures of GRK2 show that the RH, catalytic and PH domains are arranged as the three sides of an equilateral triangle. The RH domain and the carboxyl-terminal region form extensive contacts mediated by a hydrophobic patch and ionic interactions (Lodowski et al., 2003; Tesmer et al., 2005) (Figure 1.1B). While the RH domain is predominantly amino-terminal, two of its helices are

contributed by a 34 amino acid sequence that is adjacent to the carboxyl-terminal PH domain, thus placing these two regions next to each other in the folded structure (Figure 1.1A and 1.1B). GRK2 can bind to $G\alpha_{\text{guanosine triphosphate (GTP)}}$ via its RH domain and $G\beta\gamma$ via its PH domain simultaneously but with $G\alpha_{\text{GTP}}$ and $G\beta\gamma$ completely separated from each other. GRK2 binds to $G\beta\gamma$ at essentially the same site as $G\alpha_{\text{guanosine diphosphate (GDP)}}$, explaining why GRK2 competes with $G\alpha_{\text{GDP}}$ for $G\beta\gamma$ binding (Pitcher et al., 1992). This also explains how $G\beta\gamma$ can recruit GRK2 to the plasma membrane in an agonist-dependent fashion as the GRK2 binding site on $G\beta\gamma$ will only be exposed when $G\alpha_{\text{GTP}}$ is removed from this site upon heterotrimeric G protein activation.

(A) GRK2 Secondary Structure



(B) GRK2 Tertiary Structure

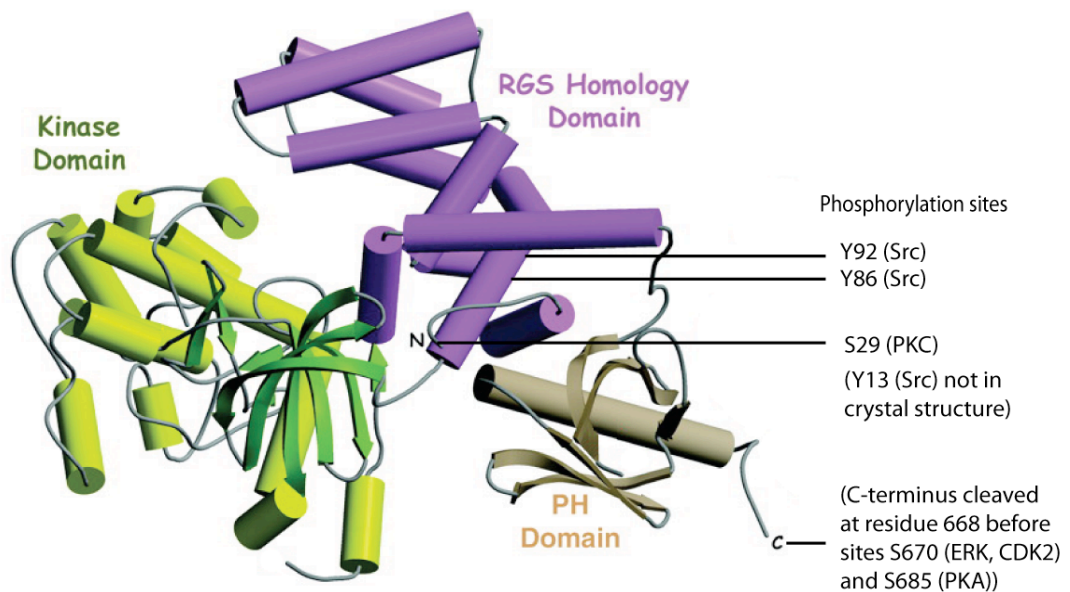


Figure 1.1. Structure of GRK2. (A) Schematic of GRK2 secondary structure with phosphorylation sites and known binding partners indicated. (B) GRK2 tertiary structure (membrane proximal view, from (Lodowski et al., 2003)) showing close association of RH and PH domains. See text or table on page 5 for details of abbreviations.

1.1.2. Desensitisation of GPCRs.

The GRKs are classically known for their ability to phosphorylate and desensitise GPCRs. Upon their activation, GPCRs promote the exchange of GDP for GTP in the $G\alpha$ subunit of their associated heterotrimeric G protein. This results in activation of the G protein and exposure of the effector binding sites on its $G\alpha$ and $G\beta\gamma$ subunits. GRK2 is recruited to agonist occupied receptors via its PH domain binding to activated $G\beta\gamma$ subunits in the presence of PIP_2 . Once bound to the activated GPCR, GRK2 is allosterically activated and subsequently phosphorylates the GPCR at serine and threonine residues usually within, either the third intracellular loop, or carboxyl-terminal tail. This triggers β -arrestin binding to the receptor, which prevents any further receptor-dependent activation of heterotrimeric G proteins. β -arrestins then initiate clathrin-coated pit-mediated receptor internalisation via recruitment of a number of proteins, including the clathrin adaptor AP2 (Pitcher et al., 1998a; Premont and Gainetdinov, 2007).

As well as instigating β -arrestin recruitment, GRK2 may also mediate receptor internalisation via recruitment of G protein-coupled receptor kinase interacting protein 1 (GIT1) and phosphatidylinositol-3-kinase (PI3K) to agonist occupied GPCRs (Premont et al., 1998). GIT proteins are thought to promote clathrin coated pit-mediated GPCR endocytosis by functioning as GAPs for the small GTPase ADP ribosylation factor 6 (Arf6), which is important in vesicle formation (Hoefen and Berk, 2006). GRK2 also directly interacts with PI3K, the recruitment of which promotes β_2 -adrenergic receptor (β_2 AR) endocytosis (Naga Prasad et al., 2002; Perrino et al., 2005), although the precise mechanism for this is poorly understood.

In some cases, GRK2 has been shown to desensitise GPCRs via β -arrestin-independent mechanisms. It can interact directly with clathrin via a clathrin box within its carboxyl-terminal region (Figure 1.1A), enabling β -arrestin-independent internalisation of the β 1-adrenergic receptor (β 1AR) (Shiina et al., 2001). In another mechanism that acts at a level downstream of receptors, the RH domain of GRK2 can interact directly with activated Gq subunits. Since the RH domain of GRK2 lacks GAP activity (section 1.1), this promotes desensitisation of Gq-mediated signalling by sequestering Gq_{GTP} subunits away from their downstream effectors such as phospholipase C β (PLC β) (Carman et al., 1999b). Notably, the interaction of GRK2 with Gq_{GTP} looks, based on its crystal structure, more like a Gq_{GTP}/effector interaction than a Gq_{GTP}/GAP interaction, suggesting that GRK2 may have additional signalling functions downstream of Gq-coupled GPCRs other than simply sequestration of Gq (Tesmer et al., 2005).

1.1.3. Regulation of GRK2-mediated GPCR desensitisation.

Desensitisation of GPCRs by GRK2 is subject to many mechanisms of regulation. Phosphorylation by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), extracellular signal-regulated protein kinase 1/2 (ERK1/2) and sarcoma virus homologue (Src) family kinases at sites labeled in Figure 1.1 as well as direct interactions with calmodulin, caveolin, and Raf kinase inhibitor protein (RKIP) are all known to play a part (Figure 1.2). PKA phosphorylation at serine 685 promotes G $\beta\gamma$ binding to GRK2, resulting in positive regulation of membrane translocation of GRK2 and GPCR desensitisation. Mutation of S685 to alanine reduces GRK2 translocation to and phosphorylation of the β 2AR in HEK-293 cells

(Cong et al., 2001). In gastric smooth muscle cells, GRK2 phosphorylation at S685 by PKA has also been shown to promote binding and sequestration of Gq_{GTP}, inhibiting PLCβ1-mediated phosphoinositide hydrolysis (Huang et al., 2007b). Thus, phosphorylation of GRK2 by PKA may play a dual role in promoting GPCR desensitisation, at least downstream of Gq-coupled GPCRs, where phosphorylation at S685 would increase GRK2 recruitment to the receptor and additionally promote GRK2-mediated Gq_{GTP} sequestration.

PKC phosphorylation at serine 29 also positively regulates desensitisation but this is thought to be due to inhibition of calmodulin binding to GRK2, which otherwise binds within the amino-terminal region to inhibit GRK2 kinase activity (Krasel et al., 2001; Pronin et al., 1997). For example, phosphorylation of GRK2 by PKC in response to activation of the angiotensin receptor is an important mechanism by which GRK2 is activated in cardiac myocytes during mechanical stretch-induced hypertrophy (Malhotra et al., 2010). The roles of GRK2 in cardiovascular disease, including pathological cardiomyocyte hypertrophy, will be discussed in detail in section 1.3. Like calmodulin, direct binding of caveolin to GRK2 also negatively regulates GPCR desensitisation. Caveolin binding sites have been mapped in the PH domain and RH domain of GRK2 (Figure 1.1) where binding negatively regulates intrinsic GRK2 kinase activity (Carman et al., 1999a).

RKIP normally binds to and inhibits rapidly accelerated fibrosarcoma associated gene 1 (Raf1) kinase activity, reducing background ERK activation in cells. However, in response to PKC phosphorylation, it performs an acutely effective switch to prolong GPCR-mediated ERK signalling by releasing its inhibition of Raf1 and moving to directly bind to and inhibit GRK2 via the GRK2 amino-terminal domain (Figure 1.1). Gβγ binding by GRK2 is not affected

suggesting that RKIP binding, like caveolin and calmodulin binding to GRK2, inhibits intrinsic GRK2 kinase activity (Lorenz et al., 2003). For example, in gastrointestinal smooth muscle cells, PKC activation downstream of muscarinic type III receptors (M3MRs) results in RKIP phosphorylation and association of RKIP with GRK2. This reduces GRK2-mediated vasoactive intestinal peptide receptor 2 (VPAC2) receptor desensitisation and increases ERK activation (Huang et al., 2007a). Thus, as well as directly phosphorylating and positively regulating GRK2 activity by reducing calmodulin binding, PKC can indirectly, via phosphorylation of RKIP, also negatively regulate GRK2 activity.

Phosphorylation of GRK2 by Src family kinases at tyrosine residues including Y13, Y86 and Y92 positively regulates desensitisation by directly increasing GRK2 kinase activity (Fan et al., 2001; Penela et al., 2001; Sarnago et al., 1999). For example, Src phosphorylation of GRK2 in response to acetylcholine binding at the muscarinic type II receptor (M2MR) increases GRK2-mediated desensitisation of VPAC2 receptors (Mahavadi et al., 2007). Similarly, GRK2 is recruited, in a G $\beta\gamma$ -dependent manner, to the epidermal growth factor (EGF) receptor (EGFR) and phosphorylated at tyrosine residues 13, 86 and 92 in response to agonist binding (Chen et al., 2008; Gao et al., 2005a). Src is not required for EGF-mediated GRK2 tyrosine phosphorylation, suggesting that GRK2 may be directly phosphorylated by the EGFR. The result of this is increased GRK2-mediated desensitisation of opioid receptors (Chen et al., 2008). The same (as well as additional) tyrosine residues are phosphorylated downstream of the platelet-derived growth factor (PDGF) receptor (PDGFR) but this results in desensitisation of the PDGFR itself (discussed in the next section).

Two of the tyrosine residues (86 and 92) phosphorylated by Src are located within the GRK2 RH domain (Figure 1.1) suggesting that tyrosine phosphorylation may also affect Gq_{GTP} binding. Indeed, constitutively active Src promotes co-immunoprecipitation of GRK2 with Gq_{GTP}, and Gq_{GTP} binds poorly to GRK2 when these residues are mutated to phenylalanines (Mariggio et al., 2006). Thus, like PKA phosphorylation of GRK2, Src phosphorylation of GRK2 may promote desensitisation of Gq-mediated signalling by sequestering Gq from its effector PLC β as well as by increasing GRK2 kinase activity. In contrast to PKA, PKC and Src phosphorylation of GRK2, which all positively regulate GRK2-mediated GPCR desensitisation, ERK1/2 phosphorylation of GRK2 and S-nitrosylation of GRK2 reduce GRK2-mediated GPCR desensitisation. Phosphorylation by ERK1/2 at serine 670 reduces intrinsic GRK2 kinase activity (Pitcher et al., 1999), while S-nitrosylation of GRK2 at cysteine 340 within the catalytic domain reduces GRK2-mediated phosphorylation of, and β -arrestin recruitment to, β -adrenergic receptors (Whalen et al., 2007). A schematic summarising the various regulatory mechanisms operating to control GRK2-mediated GPCR phosphorylation is shown in Figure 1.2.

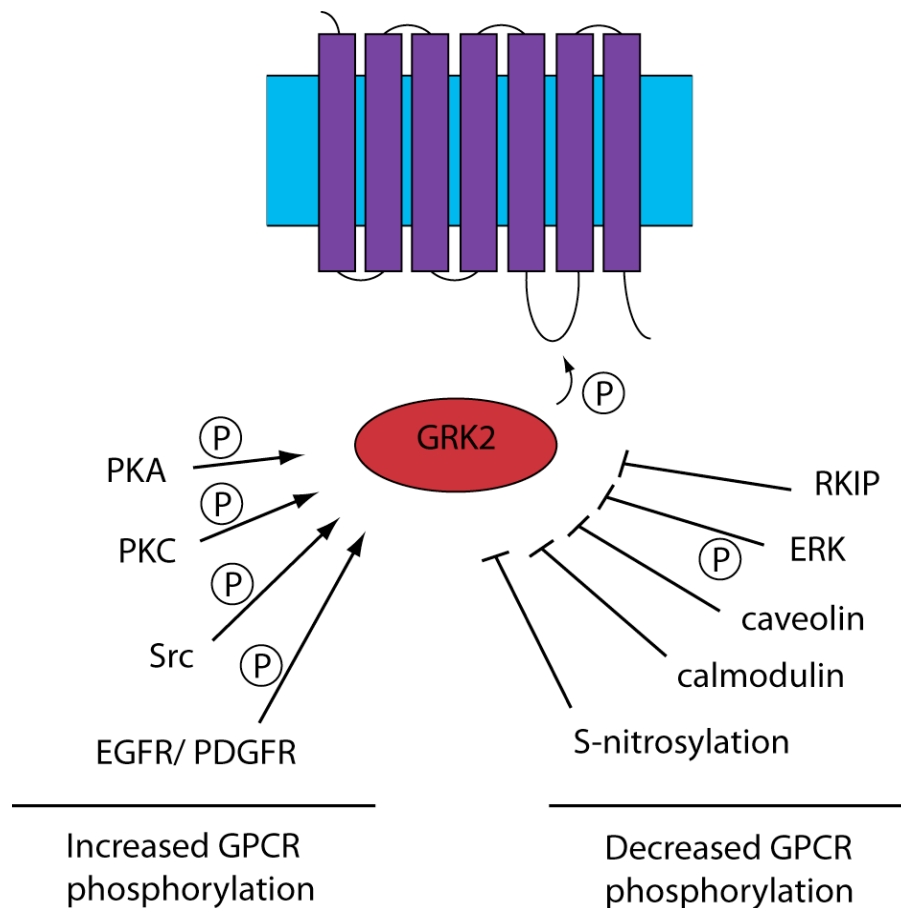


Figure 1.2. Regulation of GRK2-mediated phosphorylation of GPCRs.

GRK2-mediated GPCR desensitisation can also be regulated by modulating the levels of GRK2 in cells at the transcriptional level or by proteins that regulate its stability. For example, GRK2 transcription has been shown to increase following transforming growth factor β (TGF- β) stimulation of vascular smooth muscle (VSM) cells (VSMCs), resulting in an inhibition of angiotensin II (ANG_{II})-mediated ERK activation (Guo et al., 2009). In contrast, pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) have been shown to negatively regulate GRK2 gene promoter activity (Ramos-Ruiz et al., 2000). Degradation of GRK2 can occur via the proteasome, a process that is potentiated by β_2 AR stimulation (Penela et al., 1998), or via the non-proteasomal protease calpain (Lombardi et al., 2002). Heat-

shock protein 90 (hsp90) binding to GRK2 protects GRK2 from degradation by the proteasome (Luo and Benovic, 2003) and thus may act to stabilise basal GRK2 levels. Degradation of GRK2 by calpain has been found to account for a 50% reduction in GRK2 protein levels in lymphocytes during oxidative stress. This results in a 70% reduction in agonist-induced internalisation of the β_2 AR (Lombardi et al., 2002).

1.1.4. GRK2-mediated desensitisation of Receptor Tyrosine Kinases (PDGF and Insulin receptors).

Although best known for regulating GPCR signalling, GRK2 has also been implicated as a modulator of receptor tyrosine kinase (RTK) signalling (Hupfeld and Olefsky, 2007). In fact a surprising number of parallels can be drawn between GPCR and RTK signalling, including coupling of some RTKs to heterotrimeric G proteins (Waters et al., 2004). Akin to the well-documented agonist-dependent recruitment to GPCRs, GRK2 translocates to ligand-activated PDGFRs and phosphorylates their intracellular domains (Freedman et al., 2002). Recruitment of GRK2 to the PDGFR results in reduced receptor activation reminiscent of GRK2-mediated desensitisation of GPCRs. GRK2 is directly phosphorylated by PDGFRs at tyrosine residues including those implicated as Src phosphorylation sites (Y13, 86 and 92) but also at additional tyrosine residues. This results in increased GRK2 kinase activity (as described in the previous section), thus forming a negative feedback loop for inhibition of PDGF signalling (Wu et al., 2005). GRK2 also translocates to and phosphorylates ligand-activated EGFRs resulting in phosphorylation of GRK2 at tyrosine residues 13, 86 and 92. However, rather than

negatively regulating EGF signalling, this promotes trans-regulation of GPCRs (previous section) and may also positively regulate signalling downstream of the EGFR, at least in terms of mitogen-activated protein kinase (MAPK) activation (section 1.1.7)

The insulin receptor is one example of an RTK that signals via Gq (Imamura et al., 1999). Just as GRK2 can desensitise Gq-mediated signalling at a level downstream of GPCRs by directly interacting with and sequestering active Gq, it can also inhibit signalling downstream of the insulin receptor using its RH domain to sequester Gq_{GTP} subunits, preventing insulin-induced glucose transporter type 4 (GLUT4) translocation to the membrane (Usui et al., 2004). Thus GRK2 is involved in both kinase-dependent and kinase-independent mechanisms of desensitisation at RTKs as well as GPCRs. Note that GRK2 can also regulate insulin signalling by phosphorylating the downstream Insulin Receptor Substrate-1 (IRS-1), as discussed in the following section.

1.1.5. Non-receptor substrates of GRK2.

Besides its important and highly regulated role in desensitising receptors, GRK2 has a more complex role in regulating signalling at levels downstream of receptors, mediated by its interactions with multiple non-receptor substrates and binding partners (Figure 1.1A and Table 1.1). Thus, GRK2 is able to regulate different pathways in both positive and negative ways feeding into many aspects of cellular function (Ribas et al., 2007). In this section I will describe the non-receptor substrates of GRK2 before discussing the various non-receptor binding partners of

GRK2 in the following section. Finally I will specifically discuss the roles of GRK2 in regulating MAPK signalling in section 1.1.7.

Substrate	Effect	Reference
Insulin receptor substrate 1 (IRS-1)	Reduced insulin signalling	(Ciccarelli et al., 2011)
Smoothened	Activation of hedgehog signalling	(Chen et al., 2011)
p38	Inhibition of MAP kinase kinase 6 (MKK6)-mediated activation	(Peregrin et al., 2006)
SMADs 2 and 3	Inhibition of TGF- β signalling	(Ho et al., 2005)
Ezrin/Radixin	M1 muscarinic receptor (M1MR)-dependent membrane ruffling	(Cant and Pitcher, 2005)
Downstream regulatory element antagonist modulator (DREAM)	Inhibition of Kv4.2 potassium channel expression	(Ruiz-Gomez et al., 2007)
Epithelial Na Channels (ENaCs) and Nedd4 proteins	Disruption of Nedd4-mediated ENaC inhibition	(Dinudom et al., 2004) (Sanchez-Perez et al., 2007)
Ribosomal protein P2	Activation of translation	(Freeman et al., 2002)
Tubulin	Not known	(Carman et al., 1998)
Synucleins	Reduced inhibition of phospholipase D2	(Pronin et al., 2000)
Phosducin	Reduced binding of phosducin to G $\beta\gamma$	(Ruiz-Gomez et al., 2000)
Phosphodiesterase γ (PDE γ)	Increased ERK activation	(Wan et al., 2003)

Table 1.1. Non-receptor substrates of GRK2.

Insulin receptor substrate 1 (IRS-1).

As previously described, GRK2 desensitises Gq-mediated signalling downstream of the insulin receptor via RH domain-mediated sequestration of Gq_{GTP} subunits, preventing insulin-induced GLUT4 translocation to the membrane (Usui et al., 2004) (section 1.1.4). GRK2 also has a kinase activity-dependent role in regulating insulin signalling. Endothelin-1-induced insulin resistance in 3T3-L1 adipocytes results in part from phosphorylation and degradation of insulin receptor substrate-1 (IRS-1). Endothelin-1-induced insulin resistance is associated with diseases including type 2 diabetes and obesity. The ability of endothelin-1 to promote IRS-1 phosphorylation and insulin resistance is reversed by over-expression of kinase dead but not wildtype GRK2 (Usui et al., 2005). GRK2 interacts with (Garcia-Guerra et al., 2010) and directly phosphorylates (Ciccarelli et al., 2011) IRS-1. GRK2 levels are high in insulin-resistant cultured human adipocytes (Mayor et al., 2011), suggesting that indeed GRK2 plays an important role in mediating GPCR-dependent insulin resistance.

Of particular relevance to cardiovascular disease (see section 1.3), GRK2, which is found at high levels in the failing heart, promotes myocardial insulin resistance by directly phosphorylating IRS-1 and reducing GLUT4 membrane translocation (Ciccarelli et al., 2011). Inhibition of GLUT4 membrane translocation was completely dependent on GRK2 kinase activity suggesting that in cardiac myocytes the kinase-independent role of sequestering Gq_{GTP} subunits is not sufficient to promote insulin resistance. The reduction in glucose uptake observed in the failing heart and the increased dependence on fatty acid metabolism reduces

cardiac efficiency. This can be reversed by inhibition of GRK2 activity (Ciccarelli et al., 2011).

SMADs 2 and 3.

TGF- β is a ubiquitously expressed growth factor important in development and many diseases including cancer. Its receptor has intrinsic serine/threonine kinase activity, which phosphorylates cytoplasmic proteins called SMADs in response to agonist binding. SMADs 2 and 3 (the receptor regulated SMADs) bind to SMAD 4 in response to phosphorylation by the receptor and this allows shuttling to the nucleus where the SMAD complexes can act as co-activators or co-repressors for many different genes (Shi and Massague, 2003). Overexpression of wildtype GRK2, but not kinase dead GRK2, in cells results in reduced nuclear translocation of SMADs and reduced TGF- β -induced gene expression (Ho et al., 2005). GRK2 can phosphorylate SMADs 2 and 3 at threonine residue 197 within their linker regions. This prevents phosphorylation of SMADs 2 and 3 by the receptor at carboxyl-terminal serine and threonine residues, an event required for activation (Ho et al., 2005). TGF- β signalling is known to upregulate GRK2 at the transcriptional level, thus, GRK2 engages in a negative feedback loop to negatively regulate anti-proliferative and pro-apoptotic signalling downstream of TGF- β (Ho et al., 2005). By acting downstream of the receptor rather than at the receptor itself, GRK2 can specifically inhibit SMAD-mediated but not SMAD-independent pathways.

Epithelial sodium channels (ENaCs) and neural precursor cell expressed developmentally down-regulated 4 (Nedd4) proteins.

Epithelial sodium channels (ENaCs), which are expressed in a number of tissues including the renal collecting duct, are vital for regulating sodium and fluid homeostasis. They are tightly regulated by a negative feedback loop in which the ubiquitin protein ligases Nedd-4 and Nedd-4-2 bind to the channels in response to increased intracellular sodium and direct their internalisation and degradation (Eaton et al., 2010). Disruption of this feedback mechanism has been implicated in hypertension, a disease in which GRK2 is also known to be a determinant (see section 1.3.2). Phosphorylation of ENaCs, for example in response to hormones such as insulin, is also known to regulate their function (Eaton et al., 2010). ENaCs are an example of a non-receptor membrane substrate for GRK2 (Dinudom et al., 2004). Phosphorylation of a specific serine residue within the ENaC β -subunit disrupts the channel's negative feedback mechanism by preventing Nedd-4-2-mediated endocytosis and degradation, thus providing one potential mechanism by which GRK2 may cause hypertension (discussed further in section 1.3.2).

GRK2 has also been found to co-immunoprecipitate with and phosphorylate the Nedd-4 proteins themselves (Sanchez-Perez et al., 2007). The function of Nedd-4 phosphorylation remains unclear, however GRK2 clearly plays a complex role in epithelial sodium transport regulation. Interestingly, it has since been shown that, when transfected into mouse renal collecting duct cells, a kinase dead mutant of GRK2 can increase the basal activity of ENaCs as effectively as wildtype GRK2, suggesting that GRK2 can regulate the basal activity of these channels using a kinase-independent mechanism (Lee et al., 2011). GRK2 mutated in its RH domain

such that it cannot bind Gq fails to affect basal ENaC activity. Active Gq was found to inhibit basal ENaC activity in a Nedd-4-independent manner and this inhibition was relieved in the presence of the GRK2 RH domain (Lee et al., 2011). Thus, GRK2 can regulate epithelial sodium transport using both kinase-dependent and independent mechanisms.

Smoothened.

Members of the hedgehog family of secreted proteins bind to the 12-transmembrane protein patched. In the absence of hedgehog, patched inhibits the membrane protein smoothened (recently classified as a GPCR (Schulte, 2010)) via a poorly understood mechanism. In response to hedgehog binding, inhibition of smoothened by patched is alleviated and smoothened can signal to transcriptional pathways involved in many aspects of embryonic development (Chen et al., 2004). Phosphorylation of its carboxyl-terminal tail is known to be important for smoothened activation (at least in drosophila) (Chen et al., 2011). GRK2 has been shown *in vitro* to directly phosphorylate the carboxyl-terminal tail of smoothened at multiple sites and studies performed in mammalian cells and in zebrafish show that GRK2 can promote smoothened-mediated transcription in a kinase-dependent mechanism that may involve recruitment of β -arrestin-2 (Chen et al., 2004; Chen et al., 2011; Meloni et al., 2006). This is clearly reminiscent of the function of GRK2 at classical GPCRs, where phosphorylation of receptors and recruitment of β -arrestins can positively regulate various signalling pathways including ERK activation (DeWire et al., 2007). Knockdown of GRK2 in zebrafish results in impaired muscle and neural development while, in mammalian cells, over-

expression of GRK2 results in increased smoothened-mediated oncogenic transformation (Meloni et al., 2006; Philipp et al., 2008).

In drosophila, carboxyl-terminal tail phosphorylation of smoothened by GRK2 has also been shown to activate it but an additional, kinase-independent function of GRK2 is also proposed whereby GRK2 binds to and stabilizes the active conformation of smoothened (Chen et al., 2010b). Furthermore, hedgehog/smoothened signalling was shown to upregulate GRK2 expression, thus forming a positive feedback loop. In mammals, accumulation of smoothened in the primary cilia is vital for hedgehog signalling, a process that is thought to be facilitated by hedgehog binding to patched (Rohatgi et al., 2007). A recent study has shown that serine and threonine residues in smoothened, which are known to be phosphorylated by GRK2 in response to hedgehog, are important both for β -arrestin-2 recruitment to smoothened and for smoothened localisation to the primary cilia (Chen et al., 2011). It is proposed that GRK2 phosphorylation of smoothened in response to hedgehog binding to patched both promotes the active conformation of smoothened and the recruitment of β -arrestin-2, which in turn promotes localisation of smoothened to the primary cilia. It is not clear whether the kinase-independent mechanism of smoothened regulation by GRK2 or the upregulation of GRK2 in response to hedgehog, which have been reported in drosophila, operate in mammalian cells.

Cytoskeletal components.

Ezrin is a membrane-cytoskeleton linker protein that adopts a functional conformation for plasma membrane and actin binding in response to phosphorylation at carboxyl-terminal threonine residue 567 (Chambers and

Bretscher, 2005). GRK2 can phosphorylate ezrin at this site, maintaining it in an active conformation (Cant and Pitcher, 2005). Furthermore, GRK2 is required for ezrin-dependent membrane ruffling of human epithelial (Hep2) cells in response to muscarinic type I receptor (M1MR) activation (Cant and Pitcher, 2005), while in human embryonic kidney 293 (HEK-293) cells, GRK2-mediated ezrin phosphorylation is required for β_2 AR internalisation (Cant and Pitcher, 2005). Radixin, another member of the closely related ezrin/radixin/moesin (ERM) family of membrane-cytoskeleton linker proteins, is also phosphorylated by GRK2 at the equivalent activating threonine residue (Kahsai et al., 2009). GRK2 phosphorylation of radixin promotes membrane protrusion and motility of Madin-Darby canine kidney (MDCK) epithelial cells (Kahsai et al., 2009). Taken together, these studies demonstrate that GRK2 can positively regulate reorganisation of the actin cytoskeleton via phosphorylation of ERM proteins. Tubulin, the building block of microtubules, is also a GRK2 substrate. Phosphorylation of tubulin by GRK2 is promoted in response to GPCR stimulation but the functional relevance of tubulin phosphorylation is not known (Carman et al., 1998; Pitcher et al., 1998b). Note also that another cytoskeletal protein, the multi-functional actin binding protein α -actinin, interacts with all GRKs. It is not a substrate but rather potently inhibits GRK-mediated GPCR desensitisation (Freeman et al., 2000).

Ribosomal Protein P2.

Ribosomal protein P2 can be phosphorylated in order to regulate protein synthesis by the ribosome (Vard et al., 1997). 2-dimensional gel electrophoresis demonstrated that endogenous ribosomal protein P2 is phosphorylated in HEK-293

cells in a GRK2-dependent manner upon stimulation of the β_2 AR (Freeman et al., 2002). GRK2 phosphorylates ribosomal protein P2 at sites within its carboxyl-terminal region known to be involved in the positive regulation of protein synthesis (Vard et al., 1997). These results suggest that, following GPCR activation, GRK2 may signal to the ribosome to increase protein synthesis.

Synucleins.

The synucleins are a family of four 14 kDa proteins that are highly expressed in the central nervous system. Their normal cellular functions are not fully understood but aggregation of α -synuclein has been implicated in a number of different neurodegenerative diseases including Parkinsons disease, where it is found deposited in Lewy bodies (Paleologou et al., 2005). The synucleins are also known to interact with phospholipids and inhibit phospholipase D2 *in vitro*. GRK2 can phosphorylate α - and β -synucleins at serine residue 129, resulting in a reduced interaction of both with phospholipids and reduced synuclein-mediated inhibition of phospholipase D2 (Pronin et al., 2000). Phosphorylation of α -synuclein at serine 129 is known to promote its oligomerisation and formation of inclusion bodies (Sugeno et al., 2008). GRK2 is present in neurofibrillary tangles in Alzheimer-diseased brains (Takahashi et al., 2006), suggesting that GRK2-mediated phosphorylation of α -synuclein may be important in inclusion body formation. However, it should be noted that the synucleins are substrates for all of the GRKs and, in HEK-293 cells, GRK3 or GRK6 are more likely to be the relevant synuclein kinases (Sakamoto et al., 2009), while in Parkinson's-diseased brains, GRKs 3 and 5 (but not GRK2) are upregulated (Bychkov et al., 2008). Thus, while the synucleins

do represent a non-receptor class of substates for GRK2, it is not clear whether this is important in neuropathology.

Downstream regulatory element antagonist modulator (DREAM) and the Kv4.2 potassium channel.

Downstream regulatory element antagonist modulator (DREAM) is a neuronal calcium sensing protein that is required for trafficking of Kv4 potassium channels to the plasma membrane. GRK2 can regulate potassium channel function by phosphorylating DREAM resulting in inhibition of DREAM-mediated membrane expression of the Kv4.2 potassium channel (Ruiz-Gomez et al., 2007).

Phosducin.

Phosducin and phosducin-like protein (PhLP) are ubiquitously expressed proteins that can bind to $G\beta\gamma$, sequestering it from its downstream effectors. GRK2 can phosphorylate both of these proteins in their C-termini, regions known to be important for $G\beta\gamma$ binding (Ruiz-Gomez et al., 2000). Indeed, GRK2-phosphorylated phosducin has a 50% reduced affinity for $G\beta\gamma$ relative to unphosphorylated phosducin (Ruiz-Gomez et al., 2000). Thus, GRK2 could act to relieve the phosducin-mediated sequestration of $G\beta\gamma$ subunits however, the cellular contexts in which this may be relevant are not known.

1.1.6. Non-receptor binding partners of GRK2.

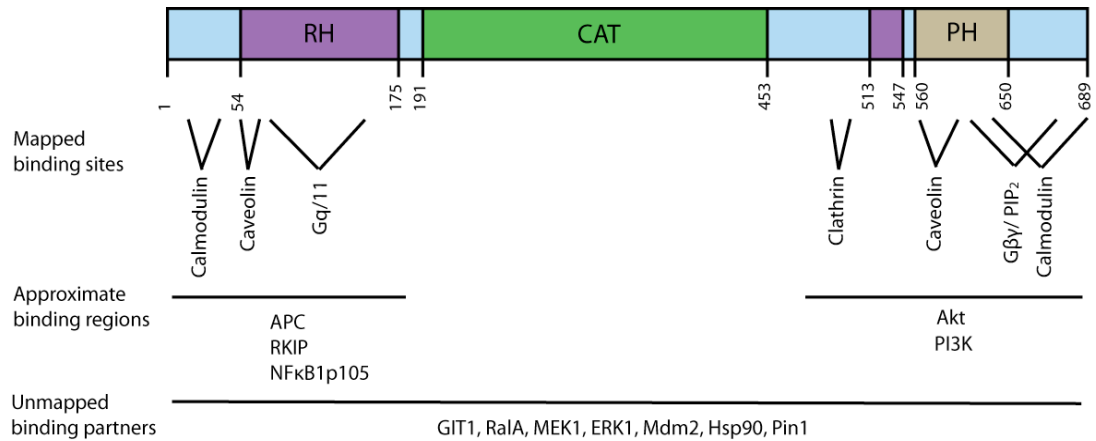


Figure 1.3. Non-receptor binding partners of GRK2. Abbreviations for and functions of the GRK2 binding proteins are described in the text.

Adenomatous polyposis coil protein (APC).

Wnt is a secreted glycoprotein that binds to and activates the GPCR frizzled. In canonical Wnt signalling, this results in release of β -catenin from a multiprotein complex containing axin and APC (adenomatous polyposis coil protein), which, in the absence of Wnt signalling, mediates degradation of β -catenin. The stabilised β -catenin can then translocate to the nucleus to activate its target genes (Smalley and Dale, 1999). The integrity of the degradation complex relies on an interaction between APC and axin, mediated by an RGS domain in axin (Wang et al., 2009). The RH domain in GRK2 is closely related to the axin RGS domain and GRK2 was found to co-immunoprecipitate with APC via its RH domain, resulting in inhibition of canonical Wnt signalling (Wang et al., 2009). In HEK-293 cells, over-expression of

GRK2 inhibits, while knock-down of GRK2 potentiates, Wnt-mediated β -catenin nuclear translocation and transcription. Surprisingly, the same effect was not observed for kinase dead GRK2. It seems that GRK2 kinase activity is required for its interaction with APC although it is not clear why this would be the case. Inhibition of GRK2 might therefore be a useful strategy for increasing canonical Wnt signalling in diseases such as osteoporosis, in which loss of this signalling pathway results in low bone mass (Wang et al., 2009). Note that the negative role of GRK2 in Wnt signalling is in complete contrast to that of GRKs 5 and 6, which are required for Wnt/frizzled signalling, reviewed in (Chen et al., 2010a).

Akt8 virus oncogene cellular homologue (Akt).

In sinusoidal endothelial cells, Akt8 virus oncogene cellular homologue (Akt) signalling activates endothelial nitric oxide synthase (eNOS). Impaired Akt activity causes reduced nitric oxide (NO) production in these cells (Liu et al., 2005). GRK2 directly interacts with Akt and inhibits its serine/threonine kinase activity (Liu et al., 2005) (Figure 1.3). After liver injury in rats, GRK2 protein levels are increased in sinusoidal endothelial cells while Akt activation and NO production are reduced (Liu et al., 2005). When GRK2 is knocked down by siRNA in these cells, Akt activity and NO production is restored. In addition, portal hypertension in response to liver injury is reduced in GRK2 heterozygous mice (Liu et al., 2005). Thus, GRK2 appears to be playing a pathological role in negatively signalling to Akt. How exactly GRK2 inhibits Akt activity is not clear but binding is shown to be via the carboxyl-terminal region of GRK2 and the authors suggest that this may displace Akt from its proper sub-cellular location (Liu et al., 2005). Interestingly,

Akt and NO are also important for protecting the myocardium from ischemia/reperfusion injury, a condition in which GRK2 levels are known to correlate with myocardial apoptosis and extent of heart injury (Brinks et al., 2010). As in portal hypertension, Akt activation and NO production were shown to inversely correlate with GRK2 levels, suggesting that inhibition of NO-mediated protection of the myocardium may be another way in which elevated GRK2 levels contribute to heart failure (discussed further in section 1.3.1).

Rat sarcoma associated gene (Ras)-related protein A (RalA).

In response to binding their agonist lysophosphatidic acid (LPA), the ubiquitously expressed GPCRs, LPA₁R and LPA₂R recruit and activate the small GTPase rat sarcoma associated gene (Ras)-related protein A (RalA), which is known to have roles in receptor endocytosis (Aziziyeh et al., 2009). RalA does not however regulate LPA₁R and LPA₂R endocytosis but rather promotes LPA-induced phospholipase C activation. Over-expression of either GRK2 wildtype or kinase dead in cells desensitises inositol phosphate production downstream of LPA₁R and LPA₂R (Aziziyeh et al., 2009). GRK2 co-immunoprecipitates with RalA in response to LPA suggesting that kinase-independent desensitisation could occur by binding and sequestering of RalA by GRK2 (Aziziyeh et al., 2009), however it is not clear to what extent sequestration of Gq_{GTP} by GRK2 plays a part in this process and the signalling implications of an interaction between RalA and GRK2 remain to be determined.

Nuclear factor kappa light chain enhancer of activated B cells (NFκB) signalling.

The Nuclear factor kappa light chain enhancer of activated B cells (NFκB) family of transcription factors are sequestered in the cytoplasm by members of the inhibitor of κB (IκB) family of inhibitory subunits. Phosphorylation of IκB results in its ubiquitination and degradation, allowing NFκB transcription factors to translocate to the nucleus where they can regulate the expression of genes involved in diverse processes including apoptosis and immune responses (Bonizzi and Karin, 2004). Patial *et al* report that NFκB signalling in response to TNF-α in Raw264.7 macrophages is inhibited by small interfering RNA (siRNA) knockdown of GRK2 and activated by over-expression of GRK2 (Patial et al., 2010). It is proposed that GRK2 binds to IκBα and is required for IκBα phosphorylation and hence NFκB activation. The mechanism by which GRK2 promotes IκBα phosphorylation remains obscure however since IκBα is not a substrate for GRK2 *in vitro*. TNFα-induced IκBα phosphorylation in Raw264.7 macrophages occurs independently of inhibitor of κB kinase β (IKKβ), thus GRK2-dependent regulation of IκBα phosphorylation is not via the indirect regulation of IKKβ activity. In a separate study Sorriento *et al* demonstrate that expression of GRK2 in endothelial cells has no effect on lipopolysaccharide (LPS)-stimulated NFκB activity. They also report that the interaction of IκBα with GRK2 is five times weaker than with GRK5, a bone fide IκBα kinase (Sorriento et al., 2008). The role of GRK2 in regulating NFκB activity via an IκBα-dependent mechanism is thus still open for debate.

GRK2 also binds to another member of the IκB family of inhibitory subunits, NFκB1p105 (www.signaling-gateway.org/data/Y2H/cgi-bin/y2h.cgi) (Parameswaran et al., 2006). Phosphorylation and degradation of NFκB1p105 by

IKK β in response to LPS releases the mitogen-activated protein kinase kinase kinase (MAPKKK) tumour progression locus 2 (TPL2), which can then activate MAPK/ERK kinase (MEK) and ERK. Deletion of GRK2 enhances LPS-induced NF κ B1p105-dependent ERK activation (Patial et al., 2011). Taken as a whole the data likely suggests that (while GRK5 may be a positive regulator) GRK2 does not regulate I κ B α -mediated NF κ B activity but does negatively regulate NF κ B1p105-mediated ERK activation. Presumably, the ability of GRK2 to bind to NF κ B1p105 via its RH domain (Figure 1.3) prevents IKK β -mediated phosphorylation and degradation of NF κ B1p105. This highlights one of many ways in which GRK2 can regulate ERK activation, as will be discussed in the next section.

Prolyl-isomerase 1 (Pin1)

GRK2 protein levels are reduced by over 50% at the G2/M transition of the cell cycle (Penela et al., 2010b). Interestingly, over-expressed wildtype GRK2 follows the same pattern as endogenous GRK2 but levels of over-expressed GRK2 with serine 670 mutated to alanine (GRK2-S/A) fail to fluctuate throughout the cell cycle. Cells over-expressing GRK2-S/A are also impaired in their ability to progress through the cell cycle (Penela et al., 2010b). Cyclin-dependent kinase 2 (CDK2) is identified as an additional kinase to ERK that can phosphorylate serine 670 (Figure 1.1) on GRK2 and in cells lacking CDK2, GRK2 levels do not fluctuate. Phosphorylated GRK2 is found to interact with the prolyl-isomerase Pin1 (Figure 1.3) and in cells lacking Pin1, GRK2 levels also fail to fluctuate. Furthermore, Pin1 over-expression promotes the degradation of GRK2 (but not GRK2-S/A) and this is inhibited in the presence of a CDK2 inhibitor but not in the presence of an ERK

inhibitor. Thus, GRK2 degradation, mediated by phosphorylation at serine 670, appears to be important for progression through the cell cycle. In this context, the authors propose that serine 670 is phosphorylated by CDK2 rather than ERK, resulting in Pin1 binding and GRK2 degradation by the proteasome (Penela et al., 2010b).

Murine double minute oncogene 2 (Mdm2)

The E3 ubiquitin ligase murine double minute oncogene 2 (Mdm2) is recruited to GPCRs by β -arrestins in response to agonist treatment (Shenoy et al., 2009). Mdm2 binds to GRK2 (Figure 1.3) and can promote its turnover in response to β_2 AR receptor stimulation (Salcedo et al., 2006). Agonist-induced co-immunoprecipitation of GRK2 with Mdm2 in the absence of β -arrestins is poor, indicating that β -arrestins are required for the interaction. β -arrestin/Mdm2-mediated GRK2 turnover also requires phosphorylation of GRK2 by Src and ERK (Nogues et al., 2011). This may provide an important mechanism for switching off GRK2-mediated signalling at agonist-occupied GPCRs.

1.1.7. Mitogen activated protein kinase (MAPK) regulation by GRK2.

The MAPKs are a family of multifunctional serine threonine kinases, which includes ERK1/2, c-Jun N-terminal kinases (JNKs) 1, 2 and 3 and p38. GRK2 has multiple roles in regulating both ERK1/2 and p38 as shown in Figure 1.4.

Negative regulation of MAPK	Positive regulation of MAPK
Kinase-Dependent Roles	
<ul style="list-style-type: none">- GPCR desensitisation.- Phosphorylation of p38.	<ul style="list-style-type: none">+ β-arrestin-mediated ERK activation at GPCRs.+ Phosphorylation of PDEγ.
Kinase-Independent Roles	
<ul style="list-style-type: none">- Interaction with NFκB1p105.- Sequestration of MEK from ERK.	<ul style="list-style-type: none">+ Interaction with the ERK scaffold GIT.

Figure 1.4. MAPK regulation by GRK2.

GRK2 can negatively regulate ERK activation at levels downstream of receptors by interacting with various signalling proteins. As described in section 1.1.6, by binding to NF κ B1p105, GRK2 can negatively regulate IKK β - and TPL2-mediated ERK activation in response to LPS in mouse peritoneal macrophages (Patial et al., 2011). Another group has found that GRK2 co-immunoprecipitates with MEK, the direct upstream activator of ERK (Jimenez-Sainz et al., 2006) and various groups have suggested that this is responsible for negative regulation of chemokine-mediated ERK activation (Jurado-Pueyo et al., 2008; Kleibeuker et al., 2008). Inhibition of chemokine-mediated ERK activation by GRK2 is independent of GRK2 kinase activity and does not result in inhibition of MEK activation, supporting a sequestering role for GRK2 at the MEK/ERK interface. More recently, GRK2, upregulated in response to TGF- β in VSMCs, was found to inhibit ANG_{II}-induced ERK activation, resulting in reduced VSMC migration and proliferation (Guo et al., 2009). Again, MEK activation was unaffected, supporting an inhibitory role at the MEK/ERK interface. In keeping with its classical role in GPCR desensitisation, GRK2 can also negatively regulate ANG_{II}-induced ERK activation by phosphorylating and internalising the receptor in HEK-293 cells (Kim et al., 2005) and *in vivo* (Eckhart et al., 2002). Thus, GRK2 can inhibit ERK activation in response to different agonists in both kinase-dependent and independent ways.

p38, another MAPK, which is activated in response to extracellular stress and inflammatory signals, is negatively regulated by GRK2 in a kinase-dependent mechanism (Peregrin et al., 2006). GRK2 phosphorylates p38 at a specific threonine residue within its docking groove, a domain important for interactions with its regulators and effectors. Phosphorylation at this site prevents activation of p38 by its upstream MAPK kinase MKK6 and altering the cellular GRK2 complement

inversely correlates with p38 activation. Thus, it is hypothesised that GRK2 may play an important role in inhibiting p38-dependent mechanisms, such as differentiation of pre-adipocytic cells (Peregrin et al., 2006).

In some circumstances, GRK2 can positively regulate MAPK activation. β -arrestins, which are recruited to GPCRs in response to receptor phosphorylation by GRK2 can, as well as directing receptor internalisation, in some cases act as scaffolds for activation of numerous signalling pathways, including JNK3 and ERK (section 1.2). β -arrestin-mediated ERK activation tends to be slower to reach maximum activity and be more sustained than early onset, transient G protein-mediated ERK activation (DeWire et al., 2007). For example, siRNA depletion of β -arrestin-1 or β -arrestin-2 in HEK-293 cells attenuates late onset ERK activation downstream of the β_2 AR receptor (Shenoy et al., 2006) and the type 1 parathyroid hormone receptor (PTH1R) (Gesty-Palmer et al., 2006) without affecting the early and transient G protein-mediated ERK activation. Since GRK2 is known to phosphorylate both of these receptors (Malecz et al., 1998; Seibold et al., 1998), they are likely to be two examples of many possible GPCRs at which GRK2 may indirectly activate ERK via recruitment of β -arrestins.

As discussed in section 1.1.3, GRK2 is recruited to the EGFR and phosphorylated at tyrosine residues 13, 86 and 92, resulting in increased GRK2-mediated desensitisation of opioid receptors (Chen et al., 2008). In addition to this RTK-mediated trans-regulation of GPCRs, GRK2 can also positively regulate signalling downstream of the EGFR. ERK activation in response to EGF in HEK-293 cells is potentiated by about 2-fold in response to over-expression of GRK2 (Chen et al., 2008; Gao et al., 2005b). Note that this is in contrast to the PDGFR where GRK2 recruitment and phosphorylation at the same sites results in reduced

PDGF signalling, at least in terms of PI3K activation (section 1.1.4) (Wu et al., 2005). GRK2 phosphorylation of the PDGF receptor promotes PDGF receptor dissociation from the PDZ domain-containing sodium/hydrogen exchanger regulatory factor (NHERF), a protein that is required for PDGF but not EGF receptor dimerisation (Hildreth et al., 2004). This may explain how GRK2 can negatively regulate PDGF but not EGF receptor signalling.

EGF-mediated ERK activation is also potentiated in HEK-293 cells by over-expression of phosphodiesterase γ PDE γ (Wan et al., 2001). GRK2 can directly phosphorylate PDE γ and over-expression of a PDE γ mutant that cannot be phosphorylated by GRK2 inhibits ERK activation in response to EGF treatment. GRK2 and activated ERK co-immunoprecipitate with a preformed complex of PDE γ and Src in response to EGF treatment but the phosphorylation-deficient mutant of PDE γ interacts poorly with GRK2 in response to EGF (Wan et al., 2003). Thus, the authors propose that phosphorylation of PDE γ by GRK2 in response to EGF functions to bring GRK2 and Src into a signalling complex, which then somehow promotes Src-mediated ERK activation.

In migrating epithelial cells, GRK2 has been shown to positively regulate sphingosine-1-phosphate (S1P)-mediated ERK activation in a kinase-independent and β -arrestin-independent mechanism (Penela et al., 2008). Over-expression of wildtype or kinase dead GRK2 promotes S1P-mediated cell migration and increases MEK and ERK activation by about 2-fold. Penela *et al* propose a model in which GRK2 is tyrosine phosphorylated within its amino-terminus in response to S1P stimulation and this promotes the interaction of GRK2 with the scaffold protein GIT, which can activate ERK via scaffolding of ras-related C3 *botulinum* substrate (Rac), p21-activated kinase (PAK) and MEK. GRK2 is subsequently phosphorylated

by activated ERK at serine 670 and this serves to disrupt the interaction between GRK2 and GIT. Thus, the dynamic phosphorylation of GRK2 at tyrosine and serine residues in response to S1P is proposed to result in a transient interaction of GRK2 with GIT and subsequent Rac/PAK/MEK-mediated ERK activation at the leading edge in order to promote epithelial cell migration. In summary, GRK2 can regulate ERK activation by various mechanisms in different cell types and downstream of different classes of receptors.

1.2. Regulation of ERK1/2 using scaffold proteins.

The very closely related (84% identical) ERK1 and ERK2 are members of the MAPK family of serine/threonine protein kinases, which also includes ERK5, JNKs 1-3 and the p38 MAPKs. ERK1/2 is activated downstream of many different RTKs and GPCRs. Over 100 ERK substrates with diverse cellular functions including proliferation, migration, apoptosis and cell adhesion have been identified (Ramos, 2008). In general, ERK1/2 is activated in response to activation of the small GTPase Ras, which recruits and activates Raf family kinases at the plasma membrane. Raf is a MAPKKK that phosphorylates and activates the MAPKKs MEK1 and MEK2 that are the direct upstream kinases for the activation of ERK1/2 (Pullikuth and Catling, 2007). One way in which specificity in ERK signalling can be achieved is by scaffolding two or more components of this kinase cascade in order to regulate the strength, duration or sub-cellular localisation of signalling (Buday and Tompa).

For example, in response to GPCR activation, β -arrestins-1 and 2 can act as scaffold proteins by directly binding to Raf1 and ERK1/2 (and indirectly to MEK1).

This results in prolonged ERK activation on signalling endosomes at the expense of ERK translocation to the nucleus. β -arrestin-2, but not β -arrestin-1, can also scaffold the three components of the JNK3 signalling cascade: apoptosis signal regulating kinase (ASK1), MKK4 and JNK3, again biasing JNK3 activation to the cytoplasm (DeWire et al., 2007). Similarly, MEK partner-1 (MP-1) can bind to MEK1 and ERK1 as well as the endosomal protein p14 in order to target ERK activation to the late endosome (Ramos, 2008), while the scaffold protein kinase suppressor of Raf (KSR) can bind to Raf1, MEK1/2 and ERK1/2 (Morrison, 2001) in order to increase ERK activation at the plasma membrane in response to growth factor stimulation (Claperon and Therrien, 2007). Some scaffold proteins act to regulate ERK activation in response to specific agonists. For example, MAPK organiser 1 (Morg-1) binds to Raf1, MEK1/2 and ERK1/2 to potentiate ERK activation in response to LPA and serum but not in response to EGF (Vomastek et al., 2004). Other ‘downstream ERK scaffolds’ rather than regulating the strength or localisation of ERK activation, can achieve even greater specificity by linking activated ERK to particular downstream effectors (Ramos, 2008). For example, phosphoprotein enriched in astrocytes 15 (PEA-15) binds to ERK and ribosomal S6 kinase 2 (RSK-2) thus targeting ERK to RSK-2 resulting in increased RSK-2 phosphorylation (Vaidyanathan et al., 2007).

1.3. GRK2 in cardiovascular disease.

Given the vital role that GPCRs play in almost all aspects of physiology, it is perhaps unsurprising that changes in the level and/or activity of GRKs has been implicated in a number of different disease states including autoimmune diseases,

neuronal disorders and cancer (Metaye et al., 2005; Penela et al., 2010a; Premont and Gainetdinov, 2007). Here I shall focus on the most studied role of GRK2, its function in cardiovascular disease.

1.3.1. GRK2 and heart failure.

Germline ablation of GRK2 results in embryonic lethality before embryonic day E15.5. The embryos display hypoplasia of the ventricular myocardium (Jaber et al., 1996), suggesting that GRK2 may be important in cardiac development. Cardiac-specific GRK2 knockout mice do however survive to adulthood (Matkovich et al., 2006), indicating that GRK2 probably has a more general developmental role rather than in cardiac development *per se*. Heterozygous adult mice expressing 50% less GRK2 than littermate controls develop normally but do display increased contractile function and sensitivity to catecholamines (Rockman et al., 1998), demonstrating that GRK2 does indeed play an important role in regulating cardiac function. Failure of the heart can result from many different disease conditions including hypertension, coronary artery disease and inherited or acquired cardiomyopathies such as hypertrophic cardiomyopathy, which are often associated with cardiomyocyte hypertrophy (Harvey and Leinwand, 2011). In adult humans, GRK2 is the most abundant GRK isoform in the heart and levels of GRK2 are elevated during heart failure (Ungerer et al., 1993). This contributes to a ‘vicious cycle’ in which poor cardiac performance provokes increased catecholamine release in an attempt to restore cardiac function but rather results in a further increase in GRK2-mediated desensitisation of β ARs, thus necessitating a further increase in catecholamine release and so on (Dorn, 2009). Inhibition of GRK2 function as a

mechanism for disrupting this vicious cycle has been extensively studied and has been shown to improve contractile function of the failing myocardium (Rengo et al., 2011).

As previously described (section 1.1), GRK2 is recruited to agonist-occupied GPCRs by binding to G $\beta\gamma$ subunits via its carboxyl-terminal PH domain. A peptide encoding the carboxyl-terminal region of GRK2 (β -adrenergic receptor kinase ct, β ARKct) can thus inhibit GRK2 recruitment and activation by competing with endogenous GRK2 for free G $\beta\gamma$ subunits. Intramyocardial injection of an adenovirus encoding β ARKct into failing rat hearts normalised catecholamine signalling, improved cardiac performance and reversed left ventricular remodeling after induction of myocardial infarction (Rengo et al., 2009), supporting a potential therapeutic role for β ARKct-mediated inhibition of GRK2. Interestingly, conditional cardiomyocyte-specific deletion of Cre/floxed GRK2 can protect against heart failure progression whether the genetic ablation is performed before, or even 10 days after, experimental myocardial infarction (Raake et al., 2008). Indicating that, as well as playing a role in the onset of heart failure, persistently high levels of GRK2 in the failing heart is also detrimental for heart function. In this regard, GRK2 may have a more complex role in the failing heart other than just uncoupling catecholamine signalling. For example, as previously described, GRK2 promotes myocardial insulin resistance by phosphorylating IRS-1 and reducing GLUT4 membrane translocation (Ciccarelli et al., 2011) (section 1.1.5). The reduction in glucose uptake in the failing heart and the increased dependence on fatty acid metabolism reduces cardiac efficiency. GRK2 also directly interacts with Akt and inhibits its serine/threonine kinase activity (Liu et al., 2005). Akt activates eNOS and Akt-mediated NO production is important for protecting the myocardium from

ischemia/reperfusion injury, a condition in which GRK2 levels are known to correlate with myocardial apoptosis and extent of heart injury (Brinks et al., 2010). Akt activation and NO production inversely correlate with GRK2 levels, suggesting that inhibition of NO-mediated protection of the myocardium may be another way in which GRK2 levels contribute to heart failure.

Recent evidence suggests that the vicious cycle of heart failure could alternatively be broken at the source of the circulating catecholamines (Lympopoulos et al., 2010). GRK2 levels are also increased in the adrenal medulla in animal models of heart failure, resulting in uncoupling of α_2 -adrenergic receptors. These receptors would normally act as presynaptic inhibitory receptors in the adrenal medulla to limit the release of adrenaline. High levels of GRK2 thus cause a chronic increase in catecholamine secretion (Lympopoulos et al., 2007). Transgenic mice with approximately 50% less GRK2 in their adrenaline producing cells have reduced circulating levels of adrenaline and improved cardiac function in heart failure induced by myocardial infarction (Lympopoulos et al., 2010). Thus GRK2 contributes to heart failure by complex mechanisms in both the myocardium and in the adrenal medulla.

1.3.2. GRK2 and hypertension.

Regulation of blood vessel radius by controlling the contractile state of VSM is an important mechanism for controlling blood pressure. Vasoconstrictory and vasodilatory GPCRs on the VSM are vital for blood pressure control and when this control is lost, hypertension can result (Brinks and Eckhart). Several studies have linked increased levels of GRK2 with hypertension in both patients and animal

models (Cohn et al., 2009; Gros et al., 2000; Harris et al., 2008). Hypertension is a highly prevalent disease associated with physiological changes in the vessel wall including excessive growth and proliferation of VSMCs and can eventually result in stroke or heart failure. To address whether GRK2 plays a causative role in hypertension, Eckhart and co-workers developed transgenic mice that specifically over-express GRK2 in their VSMCs (Eckhart et al., 2002). These mice have increased blood pressure associated with vascular thickening and die of heart failure due to left ventricular overload and subsequent cardiac hypertrophy. In contrast, transgenic mice specifically over-expressing GRK5 in their VSMCs did not exhibit vascular thickening or cardiac hypertrophy, although they were hypertensive (Keys et al., 2005). While it has been proposed that increased desensitisation of vasodilatory receptors in VSMCs might be responsible for the causative role of GRK2 in hypertension, further work from the Eckhart group has shown that increased GRK2 also results in increased desensitisation of vasoconstrictory receptors in mouse VSM (Cohn et al., 2008). Thus, GRK2, which we now know can regulate many different signalling pathways (section 1.1), may have an important function other than simply GPCR desensitisation in VSMCs to promote hypertension.

Regulation of sodium and fluid homeostasis in the kidney is another mechanism by which blood pressure can be controlled. As discussed in section 1.1.5, ENaCs expressed in the renal collecting duct are vital for controlling blood pressure in this way. GRK2 can phosphorylate both the ENaC channels themselves (Dinudom et al., 2004) and their regulatory ubiquitin protein ligases, the Nedd-4 proteins (Sanchez-Perez et al., 2007), thus attenuating a negative feedback inhibitory loop (section 1.1.5). GRK2 can also regulate the basal activity of these channels by

sequestering Gq, which otherwise acts as an endogenous ENaC inhibitor (Lee et al., 2011). Taken together, these studies demonstrate that GRK2 could also promote hypertension via its ability to enhance epithelial sodium transport in the kidney. Abberant levels of GRK2 in endothelial cells may also contribute to hypertension, at least in intrahepatic portal hypertension. Akt-activated NO production by eNOS in sinusoidal endothelial cells is required to regulate portal pressure (Liu et al., 2005). Impaired Akt activity causes reduced NO production and results in intrahepatic portal hypertension. GRK2 directly interacts with Akt and inhibits its serine/threonine kinase activity (Liu et al., 2005). After liver injury in rats, GRK2 protein levels are increased in the sinusoidal endothelial cells, while Akt activation and NO production are reduced. When GRK2 is knocked down by siRNA in these cells, Akt activity and NO production is restored. In addition, portal hypertension in response to liver injury is reduced in GRK2 heterozygous mice (Liu et al., 2005). This is thought to be due to binding and sequestering of Akt by GRK2 away from eNOS (section 1.1.6).

1.4. RhoA and hypertension.

The Rho family of small GTPases forms 1 of 5 branches (Ras, Rho, Rab, Ran and Arf) of the Ras superfamily of small monomeric GTPases, which are homologous to the α -subunit of heterotrimeric G proteins. The Rho family itself comprises 20 members, divided into eight subfamilies, including the Rac, Cdc42 and RhoABC subfamilies (Heasman and Ridley, 2008; Jaffe and Hall, 2005). Most Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form, a process that is regulated by guanine nucleotide exchange factors (GEFs),

GAPs and guanine nucleotide dissociation inhibitors (GDIs). GEFs activate Rho signalling by promoting the release of GDP and subsequent binding of GTP, which is at a higher concentration in cells. GAPs and GDIs negatively regulate Rho signalling by promoting GTP hydrolysis or by cytosolic sequestration of Rho GTPases respectively.

Rho GTPases can be activated downstream of numerous GPCRs (Bhattacharya et al., 2004) and RTKs (Schiller, 2006). Downstream of GPCRs, RhoA can be activated via direct interaction of $G_{12/13}$ or $G_{q/11}$ family members with GEFs. For example, $G_{12/13}$ can recruit RGS domain-containing GEFs such as PDZ-RhoGEF, Leukemia-associated Rho GEF (LARG) and p115-RhoGEF (Fukuhara et al., 2000). Various GEFs have also been shown to mediate activation of RhoGTPases downstream of RTKs. For example, the Rho GEF Vav-2 is phosphorylated and activated downstream of the EGFR (Pandey et al., 2000) and the VSM-specific Rho GEF, vsm-RhoGEF, is tyrosine phosphorylated and activated downstream of the ephrin receptor EphA4 (Ogita et al., 2003). The RGS domain-containing Rho GEF, LARG, which is activated by $G_{12/13}$ coupled GPCRs is also recruited to the RTK insulin-like growth 1 receptor (IGF1R) in order to activate Rho (Taya et al., 2001).

When bound to GTP and activated, RhoA interacts with its downstream effectors via Rho-binding domains (RBDs) (Fujisawa et al., 1998). There are at least three classes of RBDs found in different Rho effectors including the ROCK class (Class II), the Citron class (Class III) and the HR1 Rho-binding repeats (Class I). HR1 repeats were first identified in the protein kinase N (PKN) family of serine/threonine protein kinases but have since been found in other effectors including raphilin and rhotekin (Hutchinson et al., 2011). PKN, raphilin and

rhotein all have three HR1 repeats in their amino-termini, although it seems that, at least for PKN, only the first of the three is strictly required for Rho binding (Owen et al., 2003). For PKN, the first and the second repeats have also been shown to bind to Rac.

The Rho GTPases are best known for their role in the regulation of cytoskeletal dynamics. Rac and Cdc42 promote the formation of lamellipodia and filopodia respectively (Jaffe and Hall, 2005). RhoA, B and C are best known for their role in regulating stress fibre formation via interactions with many downstream effectors including mammalian diaphanous homologue 1 (mDia1) and Rho-associated kinase (ROCK), which promote actin filament elongation and organisation (Jaffe and Hall, 2005). RhoABC signalling can also regulate gene expression by activating the serum response factor (SRF) and is involved in regulation of other cellular functions including endosomal trafficking and diseases including cancer (Heasman and Ridley, 2008). One important tool in elucidating the function of the RhoABC subfamily of RhoGTPases has been the C3 transferase. C3 is an exoenzyme purified from *Clostridium botulinum*, which selectively ADP-ribosylates and inactivates RhoA, B and C (Sahai and Olson, 2006). C3 is used in the course of this thesis and its production is described in section 2.11.6.

Elevated RhoA activity/expression is associated with hypertension (Loirand and Pacaud, 2010). There are a number of important mechanisms by which RhoA may regulate blood pressure. Most notably, it is an important regulator of VSM contraction (Puetz et al., 2009). NO production in endothelial cells (Wolfrum et al., 2004) and catecholamine production by the sympathetic nervous system (Ito et al., 2003) are also modulated by RhoA signalling. Several animal models of hypertension have highlighted the importance of RhoA. For example, spontaneously

hypertensive rats (Denniss et al., 2010) and ANG_{II}-induced hypertensive rats (Jin et al., 2006; Seko et al., 2003) display increased activity and expression levels of the small GTPase.

1.5. Aims of this thesis.

Given the number of non-GPCR substrates and binding partners emerging for GRK2, its role in regulating signalling in cells is becoming increasingly complex. As well as desensitising GPCRs, GRK2 is now known to regulate signalling from different classes of receptor in both positive and negative ways, while also regulating signalling pathways by various mechanisms at levels downstream of receptors. Thus, a further understanding of the regulation of GRK2 functions in health and disease is an important goal. In this thesis, I identify regions homologous to Rho-binding repeats within the catalytic domain of GRK2 and explore the hypothesis that the small GTPase RhoA may be a novel binding partner for GRK2. I then aim to understand how Rho may affect the function of GRK2. I demonstrate that RhoA_{GTP} binding to GRK2 promotes the interaction of GRK2 with Raf1, MEK1 and ERK2, the three components of the ERK MAPK cascade. Finally, I aim to investigate a potential novel role for GRK2 as a Rho-activated ERK MAPK scaffold protein and ask whether this may have implications in the highly prevalent disease hypertension, with which GRK2 and Rho have both been associated.

2. Materials and Methods

Tissue culture dishes were obtained from Nunc, culture medium from Gibco BRL, plasticware from Falcon or Sterilin and other reagents from Sigma, unless otherwise stated. Angiotensin_{II} (ANG_{II}) is human (sigma, A9525) and EGF is purified from murine submaxillary gland (Sigma, E1257). Antibodies, buffer ingredients and cDNAs referred to in this thesis are listed in tables 2.1-2.3.

2.1. Human Embryonic Kidney-293 (HEK-293), COS-7 fibroblast-like (COS), Human Epithelial (Hep2) and Mouse Embryonic Fibroblast (MEF) cell culture.

HEK-293, COS, Hep2 and MEF cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum (FCS), penicillin (100 IU/ml)/ streptomycin (100 ug/ml) and glutamine (2 mM). Cells were maintained in a humidified incubator at 37°C, 5% CO₂. Confluent monolayers were passaged every 3-4 days by trypsinising and replating at a ratio of 1:10. **Cell freezing and thawing:** To freeze cell stocks, two 70% confluent 9 cm plates were trypsinised and resuspended per 1 ml of freezing medium (DMEM, 20% FCS, 10% dimethylsulphoxide (DMSO) and penicillin (100 IU/ml)/ streptomycin (100 ug/ml)). After 1 week at -80°C, cells were transferred to liquid nitrogen. To recover stocks, aliquots were rapidly thawed at 37°C and plated onto a 9 cm plate in 15 ml culture medium.

2.2. HEK-293, COS, Hep2 and MEF cell transfection.

HEK-293, COS, Hep2 and MEF cells were split 24 h before transfection using Fugene HD (Roche). cDNAs were added to 100 μ l of serum-free DMEM in a 1.5 ml tube. 3 μ l of Fugene for every 1 μ g DNA was added to each tube, mixed gently and incubated at room temperature for 15 min. The media/cDNA/Fugene mixes were then added drop-wise to 50% confluent cells and between 24 and 72 h was allowed for expression.

2.3. VSMC isolation, culture and nucleofection.

VSMCs were isolated from rat aortas by enzymatic dissociation as follows. The aorta was removed from a ~300 g rat and placed in 50 ml Zero Calcium solution (see table 2.2 for buffers, page 74) on ice. The aorta was moved to a dish containing cold Zero Ca^{2+} solution and fat and connective tissue was stripped away by sliding tweezers along the length of the aorta. The resulting tube of smooth muscle was cut into ~1 mm rings and incubated in pre-warmed Low Ca^{2+} solution at 34°C for 10 min. The tissue was then moved using forceps into enzyme solution (2 ml Low Ca^{2+} /BSA solution with 2.1 mg Papain, 1.8 mg dithioerythritol (DTE)) and incubated for 1 h at 34°C. Now, in the tissue culture hood, tissue was moved with forceps into Low Ca^{2+} /bovine serum albumin (BSA) solution and washed 3 times in Low Ca^{2+} /BSA solution before being transferred into 1 ml of culture media (DMEM, 2 mM glutamine, 10% FCS (Gibco), penicillin 100 IU/ml/ streptomycin 100 μ g/ml). The tissue was then triturated using a plastic 3 ml pipette (with tip cut) until media became cloudy with cells. After removal of remaining solid tissue, cells were

transferred to a 6 cm plate. Cells were maintained at 37°C, 5% CO₂ in humidified conditions and culture medium was changed every 3 days. **For nucleofection**, the indicated concentrations of anti-GRK2 siRNA (5'-GCAGGUACCUCCAGAUCUCtt-3') (Applied Biosystems) or scrambled control were nucleofected using Amaxa Biosystems primary smooth muscle cell nucleofection kit and nucleofector according to manufacturer's instructions (programme D-33). Half of an 80% confluent 9 cm plate was used per cuvette and the cells from each cuvette were replated into 8 wells of a 12-well plate.

2.4. Co-immunoprecipitation (IP).

Cells in 9 cm dishes were transfected as indicated. 48 h later they were washed twice with cold tris-buffered saline (TBS) on ice and lysed by addition of 1 ml cold GTPase lysis buffer. A cell scraper was used to remove cell material from the dishes and lysates were transferred to a 1.5 ml tube. After 15 s sonication (Branson Sonifier 450, setting 6), lysates were cleared by centrifugation at 16,000g (13,000 rpm in Hettich bench top centrifuge Mikro 20) for 5 min and cell lysates were transferred to a clean tube. Protein concentrations were determined using the BioRad protein assay according to manufacturer's instructions and lysates were stored at -20°C. Volumes of lysates containing 250 µg protein were incubated with the indicated antibody for 1 h rotating at 4°C. Protein A/G sepharose beads (Santa Cruz) were washed in GTPase lysis buffer and 30 µl of a 50% suspension was added to each sample before incubation for a further 1 h rotating at 4°C. Beads were then spun down at 200g (1500 rpm in Hettich bench top centrifuge Mikro 20) for 1 min, the supernatant aspirated and 1 ml GTPase lysis buffer added to wash. The wash

cycle was repeated 3 times before adding 25 μ l sodium dodecyl sulphate (SDS) reducing buffer to each sample ready for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). **For co-immunoprecipitation of phospho-ERK**, the same protocol was used but cells were serum-starved overnight in serum-starving media (DMEM, 0.1% BSA, 10 mM HEPES pH 7.2 and penicillin (100 IU/ml)/streptomycin (100 μ g/ml)), treated as indicated and harvested with Buffer B in place of GTPase buffer.

2.5. ERK activation assay.

HEK-293 cells were transfected as indicated in 9 cm dishes on day 1, split 1:2 onto 12-well plates on day 2, serum-starved in serum-starving media overnight on day 3-4 and treated as indicated and harvested on day 4. Harvesting was achieved by washing the cells with cold TBS and adding 200 μ l SDS reducing buffer directly to each well. Lysates were transferred to 1.5 ml tubes, heated at 60°C for 10 min and 50 μ l of each sample was run on an SDS-PAGE gel to analyse levels of phospho- and total- ERK or stored at -20°C. Films were scanned using a GS800 densitometer and bands quantified using QuantityOne (BioRad). After subtraction of background, ERK activation was calculated by dividing values for phospho-ERK by total ERK for each sample. P-values for statistical significance were generated using the standard student's t-test by comparing control (untreated) with agonist treated samples.

2.6.VSMC thymidine incorporation.

Nucleofected VSMCs in 12-well plates were serum-starved in serum-starving media and treated as indicated in the presence of 3 uCi/ul [³H]-thymidine (PerkinElmer, 20 Ci/mMole). Cells were lysed in 1 ml 1% SDS and transferred to pyrex tubes. 2 ml Phosphate-buffered saline (PBS) was used to wash out wells and transferred to tubes. After addition of 3 ml ice cold 15% trichloroacetic acid (TCA, VWR), tubes were vortexed and incubated on ice for 5 min. Samples were then filtered through pre-wet 25 mm circular-1822-025 Whatman filters (tubes were washed out with 5 ml ice cold 5% TCA) before washing of the filters twice with 10 ml ice cold 5% TCA. Filters were dried by rinsing with absolute ethanol and transferred to scintillation vials with addition of 6 ml Ultima Gold scintillation fluid (PerkinElmer). Incorporated [³H]-thymidine was counted for 4 min per sample using a Packard Tri-Carb liquid scintillation analyser.

2.7. Direct binding assay.

Full length GRK2 (flg-GRK2) or GRK2 catalytic domain (flg-CAT) was *in vitro* translated using the Promega quick-coupled transcription/translation system according to manufacturer's instructions with addition of 2 ul [³⁵S]-methionine (Perkin Elmer, 1000 Ci/ mMole) per reaction. 10 µl of the *in vitro* translation (IVT) product was then incubated with 10 µg purified GST or GST-RhoA-V14 on glutathione sepharose beads (GE Healthcare) in 200 µl IVT buffer for 1 h rotating at 4°C. Beads were then washed 4 times in IVT buffer before adding 25 µl SDS

reducing buffer to each sample ready for analysis by SDS-PAGE and autoradiography. Samples were run alongside 1 ul of the IVT product input.

2.8. Inositol 1,4,5-trisphosphate (IP₃) assay.

24 h after transfection on 6-well plates, cells were washed with PBS and culture medium was replaced with labelling media containing [³H]-inositol (2 uCi/ml) (American Radiolabeled Chemicals, 30 Ci/mMole). A further 24 h later, cells were washed twice with PBS and HEPES buffer, and treated with or without ANG_{II}, for 30 min. Cells were placed on ice, media aspirated and 500 ul methanol added to each well. Cells were scraped and transferred to a 15 ml tube before addition of a further 500 ul of methanol to each well to collect any remaining cell material for transfer to the 15 ml tube. 1 ml water and 1 ml chloroform was added to each tube and vortexed. 100-200 mesh Dowex 1-X8 (formate form) columns (BioRad) were prepared by washing 3 times with 2 ml 2 M ammonium formate/0.1 M formic acid followed by 3 more 2 ml washes with water. The aqueous phases of the cell preparations were transferred from the 15 ml tube onto the columns. After 3 washes with 2 ml water and 3 with 5 mM sodium tetraborate/60 mM sodium formate, columns were transferred to stand in scintillation vials. Inositol phosphates were eluted with 3x 1 ml 1 M ammonium formate/0.1 M formic acid and 5 ml Ultima flo AF scintillation fluid (Perkin Elmer) was added ready for counting in a Packard Tricarb liquid scintillation counter. 5 ml Ultima gold scintillation fluid (Perkin Elmer) was added to the remaining chloroform phase in the 15 ml tubes to count total inositol incorporated into lipid. IP₃ production was calculated as labelled inositol in the aqueous phase (inositol phosphates) divided by counts from the

chloroform phase and was expressed as arbitrary units (AUs). The percentage desensitisation of IP₃ production in the presence of GRK2 was calculated by taking the difference in IP₃ production in the presence versus the absence of GRK2 transfection, divided by IP₃ production in the absence of GRK2.

2.9. Immunofluorescent labeling.

Cells plated on coverslips were fixed in 4% paraformaldehyde (TAAB)/PBS for 20 min before quenching for 10 min in 0.27% NH₄Cl/0.37% glycine in PBS. 1% BSA (First Link UK Ltd.)/0.2% saponin/PBS was then added for 30 min to block and permeabilise fixed cells. Primary antibody incubations were performed for 1 h at RT or at 4°C overnight in 1% BSA/0.2% Saponin. Cells were subsequently washed 3 times with 1% BSA/0.2% Saponin before incubation with the appropriate secondary antibody for 45 min at room temperature (RT). After 3 more washes in 1% BSA/0.2% Saponin and 2 in PBS, coverslips were mounted on slides in 90% glycerol (Sigma)/PBS/3% N-propyl-galate (Sigma). Confocal images were taken at RT with Leica ACS Apo 40x and 63x oil immersion lenses. A Leica TCS SPE confocal microscope and LAS AF software was used to acquire the images.

2.10. GRK2 kinase assay.

Kinase assays were performed in kinase assay buffer containing 50 uM [γ ³²P]-adenosine triphosphate (ATP) (~6000 cpm/ pmol) in a total volume of 25 ul using 1 ug purified rod outer segments (ROS) as a substrate. COS cells were transfected as indicated and 48 h later were lysed in 1 ml of kinase assay lysis buffer. Lysates were normalised for GRK2 expression as assessed by western blot

and incubated at 30°C for 10 min under bright illumination or in the dark (control) with the labelled ATP and ROS. Reactions were stopped by addition of an equal volume of SDS reducing buffer, and electrophoresed on 10% SDS-PAGE. The dried gels were analysed using a phosphorimager (BioRad molecular imager FX) and bands quantified using QuantityOne against a standard curve of pixels per known amount (pmoles) of [³²P]-ATP spotted onto paper.

2.11. Other techniques.

2.11.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed using the Hoefer Scientific Instruments (HSI) vertical slab gel unit SE 600 gel system. A 10% separating gel was cast between two glass plates by polymerising 30% (v/v) acrylamide in 0.375 M Tris-HCl pH8.8 and 1% SDS, using 0.3% (v/v) ammonium persulphate and 0.07% (v/v) N, N, N', N'-tetramethyl-ethylenediamine (TEMED). A 4% stacking gel was cast above the separating gel by polymerising 4% (v/v) acrylamide in 0.12 M Tris-HCl pH6.8 and 1% SDS, using 0.1% (v/v) ammonium persulphate and 0.1% (v/v) TEMED. Samples were prepared by addition of 25 ul SDS reducing buffer to 20 ug of lysate or immunoprecipitate and heated at 60°C for 10 min. ERK activation assay samples were already in SDS reducing buffer. Samples were loaded onto the gels and the gels were subjected to a constant voltage of 300 V for 1 h in gel running buffer. For direct binding experiments, radioactive gels were dried onto 3mm Whatman paper overnight and exposed to film at -80°C for 48 h.

2.11.2. Western blotting and immunodetection.

Proteins separated on SDS-PAGE gels were transferred onto nitrocellulose membrane (Hybond-ECL, Amersham) using a semi-dry electrophoretic transfer unit (Scie-plas, V20-SDB). The gel and membrane were soaked in transfer buffer, placed between 6 soaked pieces of 3mm Whatman paper and subjected to a constant current of 0.8 mA/cm² membrane for 1 h 45 min. Membranes were then incubated in blocking buffer for 1 h at room temperature followed by incubation for 1 h with the appropriate primary antibody diluted in blocking buffer. Following 5 washes over a 30 min period in tween/tris-buffered saline (TTBS), a horseradish peroxidase-conjugated secondary antibody (Amersham), diluted in TTBS, was added to the immunoblots for 1 h. The blots were then washed as before. Sufficient enhanced chemiluminescence (ECL) reagent (Amersham) was added to cover the membrane and incubated for 1 min. Bound antibody was detected by exposing the immunoblot to film (Biomax MR, Kodak) for the required time and the film was developed using an Agfa automatic film processor. For quantification of GRK2 fragment binding to RhoA-V14 and MEK1, bands were quantified using QuantityOne and amount GRK2 co-immunoprecipitated per unit RhoA-V14/MEK1 was plotted relative to full length GRK2.

2.11.3. Coomassie staining of SDS-PAGE gels.

To stain proteins, the SDS-PAGE gel was covered in Coomassie stain, heated for 1 minute at 750W in a microwave and allowed to cool at RT on a shaker.

The gel was then destained using Coomassie de-stain until proteins were visible and identifiable by comparison to molecular weight markers.

2.11.4. Bacterial transformation and plasmid DNA purification.

TOP10 (Invitrogen) chemically competent *E. coli* were thawed on ice and mixed by hand. 20 µl of cells were aliquoted into a pre-chilled 1.5 ml Eppendorf tube containing 50 ng of plasmid cDNA and incubated on ice for 30 minutes. The bacteria were then heat shocked at 37°C for 5 min before incubation on ice for 2 min. 1 ml pre-warmed super-optimal broth with catabolite repression (SOC) medium (Invitrogen) was added and the bacteria were incubated at 37°C for 1 h with shaking at 200 rpm (Kuhner ISF-1-W bacterial incubator). The bacteria were then plated onto Luria Broth (LB) agar plates containing 100 µg/ml ampicillin or 25 µg/ml kanamycin as appropriate and incubated at 37°C overnight. The next day, single colonies were picked from the plate and grown in 500 ml of LB medium containing 100 µg/ml ampicillin or 25 µg/ml kanamycin as appropriate overnight at 37°C with shaking at 200 rpm (Kuhner ISF-1-W bacterial incubator). The next day, the bacteria were pelleted and the plasmid DNA was extracted using a QIAprep Spin Maxiprep kit (QIAGEN). The concentration of the plasmid DNA was determined by measuring A_{260} using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech).

2.11.5. GST-Rho fusion protein purification.

BL21 (Invitrogen) *E. coli* were transformed and single colonies picked as described for TOP10 above. 100 ml starter cultures were diluted 1/10 in RT LB and incubated at 37°C with the appropriate antibiotics for 1 h with shaking at 200 rpm. The cultures were then moved to 20°C for 1 h with shaking at 200 rpm before addition of 0.2 mM isopropyl- β -D-thio-galactoside (IPTG) for 18 h to induce fusion protein expression. After pelleting, the bacteria were resuspended in 10 ml Fusion Protein Lysis Buffer. Lysates were sonicated on ice 3 times for 30 sec with a 1 min break in between each burst. After clearing the lysates by centrifugation for 20 min at 20,000g, 500 μ l of 50% glutathione sepharose beads (GE healthcare, washed in Fusion Protein Lysis Buffer) was added and incubated for 1 h at 4°C on a rotating wheel. The beads were then washed 3 times with Fusion Protein Lysis Buffer (with no PMSF) and 10 μ l was added to 25 μ l SDS reducing buffer to dissociate protein from beads and run on an SDS-PAGE gel with BSA standards and coomassie stained to determine fusion protein purity and concentration.

2.11.6. Production of protein translocation domain (PTD)-C3 exoenzyme, Rho inhibitor.

Recombinant glutathione S-transferase (GST)-protein translocation domain (PTD)-C3 was made in *E.Coli* BL21. Protein expression was induced in 1 l cultures with 0.3 mM IPTG for 3h at 32°C. Cell pellets were lysed in 10 ml C3 lysis buffer and snap frozen. After thawing and sonicating 3 times for 30 s on ice (Branson Sonifier 450, setting 6), lysates were cleared by centrifugation at 10,000g, 4°C for

20 min. The supernatant was incubated with 500 μ l glutathione sepharose beads (GE Healthcare), pre-washed in C3 lysis buffer, for 2 h at 4°C. Beads were washed 3 times with C3 lysis buffer and twice with thrombin buffer before incubation with 30 units thrombin, in 1 ml thrombin buffer, overnight at 4°C. The supernatant was removed, beads washed with thrombin buffer to recover all protein and solution incubated with *p*-aminobenzamidine beads for 1 h. The protein concentration of the supernatant was determined and assessed for purity by SDS-PAGE analysis. The purified C3 was snap frozen and used at 0.5 μ M for the times indicated in cell culture media.

2.11.7. Purification of ROS.

ROS were prepared from dark-adapted bovine retinas. 50 retinas were suspended in 50 ml of ROS buffer A, homogenised on ice and centrifuged at 2,000g for 5 min. The supernatant, containing the ROS, was diluted with 2 volumes of 10 mM Tris-acetate, pH 7.4, and centrifuged as above. The crude ROS pellets were resuspended in ~30 ml of ROS buffer B and homogenised again on ice and then further purified on a sucrose gradient by centrifugation in a SW-28 swinging bucket rotor at 57,000g (See ROS sucrose gradient in buffers table 2.2). The interface between 0.84 and 1.00 M sucrose was collected and diluted 1:1 with ROS buffer A without sucrose, and sedimented at 48,000g for 30 min. The pellet was resuspended in 10 ml ROS buffer A without sucrose and centrifuged as before. The resulting pellet was then resuspended in 10 ml buffer A, centrifuged at 68,000g before resuspending the pellet again in 15 ml 4 M urea in 10 mM HEPES (pH 7.0) to remove endogenous rhodopsin kinase and left on ice for 20 min. The ROS were then

diluted with four volumes of ROS buffer C and centrifuged as before. The pellet was resuspended in 10 mM HEPES (pH 7.0), re-centrifuged as before, the pellet resuspended in 10 ml HEPES buffer (pH 7.0) and homogenised. ROS are approximately 90% pure at the end of this procedure.

Table 2.1. Primary antibodies.

Antigen	Source	Product number	Dilution for Western	Dilution for Immuno- fluorescence	Volume for IP (ul)
Myc	Mouse	Millipore 05-724	1:1000		3
Myc	Rabbit	Sigma C3956	1:500		3
GRK2-3	Mouse	Millipore 05-465	1:1000	1:200	3
GRK2	Rabbit	Santa Cruz SC-562	1:500		5
HA	Rat	Roche 1867423	1:1000		3
Rho	Mouse	Santa Cruz SC-418	1:500		5
Flg	Mouse	Sigma F3165	1:1000		2
GST	Goat	Amersham 27457701	1:1000		3
GFP	Mouse	Roche 11814460001	1:1000		3
PI3K	Rabbit	Santa Cruz SC- 7177	1:500		5
Gq	Rabbit	Santa Cruz SC-393	1:500		5
MEK	Mouse	Santa Cruz SC- 6250	1:500		5
ERK	Rabbit	Sigma M5670	1:20,000		2
Raf	Rabbit	Santa Cruz SC-277	1:500		5
p-ERK	Rabbit	Cell Signalling 9401	1:1000	1:200	
GRK4-6	Mouse	Upstate 05-466	1:1000		3

Anti-mouse, anti-rat and anti-rabbit IgG HRP-conjugated secondary antibodies were from Amersham Biosciences (sourced from sheep, 1:2500). Anti-goat HRP-conjugated secondary was from DAKO (sourced from rabbit, 1:2000).

Table 2.2. Buffers.

Buffer Name	Ingredients
Blocking buffer	5% skimmed milk powder made up in TTBS
Buffer B	50 mM Tris pH 7.2 150 mM NaCl 7.5 mM MgCl ₂ 1% TX-100 40 µg/ ml PMSF 1 mM benzamidine 100 µM Na ₃ VO ₄ 10 mM β-glycerol phosphate 20 mM NaF
Coomassie stain	40% MeOH 10% acetic acid 0.05% Brilliant Blue R
Coomassie de-stain	40% MeOH 10% acetic acid
C3 lysis buffer	5 mM MgCl ₂ 1 mM DTT 40 ug/ ml PMSF made up in TBS
Fusion Protein Lysis Buffer	50 mM Tris pH 7.5 50 mM NaCl 5 mM MgCl ₂ 1 mM DTT and 1mM PMSF added immediately before use
Gel running buffer	2 M glycine 250 mM Tris-HCl 30 mM SDS

GTPase lysis buffer	10% glycerol 50 mM Tris-HCl pH8 150 mM NaCl 1% TX-100 2mM EDTA 40 ug/ ml PMSF 1 mM benzamidine
HEPES buffer	137 mM NaCl 2.7 mM KCl 20 mM HEPES 2 mM MgCl ₂ 1 mM CaCl ₂ 1 mg/ ml glucose 1 mg/ ml BSA + 10 mM pre-frozen LiCl prior to use
IVT Buffer	50 mM Tris pH 7.5 100 mM NaCl 10mM MgCl ₂ 0.5% NP40
Kinase Assay Lysis Buffer	20 mM HEPES pH 7.2 250 mM NaCl 10 mM EDTA 0.02% Triton X-100 40 ug/ ml PMSF 1 mM Benzamidine
Kinase Assay Buffer	20 mM Tris pH 7.5 2 mM EDTA 10 mM MgCl ₂ 1 mM DTT
Labelling media	10% dialysed FCS penicillin (100 IU/ml) streptomycin (100 ug/ml) made up in Media 199

Low Calcium Solution	Zero Ca solution with 0.1 mM CaCl_2
Low Calcium/ BSA Solution	Low Ca solution with 18 mg BSA/ 20 ml
PBS	137 mM NaCl 2.7 mM KCl 4.3 mM Na_2HPO_4 1.47 mM KH_2PO_4 Adjust to a final pH of 7.4.
ROS Buffer A	34 % Sucrose (w/w) 65 mM NaCl 2 mM MgCl_2 10 mM Tris pH 7.4 0.1 mM EDTA 500 μM PMSF 1 μM Leupeptin 1 μM Pepstatin
ROS Buffer B	0.77 M Sucrose 1 mM MgCl_2 10 mM Tris pH 7.4
ROS Sucrose gradient	10 ml: 0.84 M Sucrose 2 mM MgCl_2 10 mM Tris pH 7.4 0.1 mM EDTA 15 ml: 1 M Sucrose 2 mM MgCl_2 10 mM Tris pH 7.4 0.1 mM EDTA
SDS reducing buffer	25 mM Tris-HCl pH6.5 10% glycerol 8% SDS 5% β -mercaptoethanol a few grains of Brilliant Blue G

Serum Starving Media	DMEM 0.1 % BSA 10 mM HEPES pH 7.2 100 IU/ ml penicillin 100 ug/ ml streptomycin
TBS	25 mM Tris-HCl pH7.4 150 mM NaCl
Thrombin buffer	1 mM MgCl ₂ 1 mM CaCl ₂ 1 mM DTT made up in TBS
Transfer buffer	0.05 M Tris-HCl 0.04 M glycine 0.01 M SDS 20% MeOH
TTBS	50 mM NaCl 20 mM Tris-HCl pH8.0 0.1% Tween-20
Zero Calcium Solution	5.4 mM KCl 137 mM NaCl 0.44 mM Na ₂ HPO ₄ 0.42 mM NaH ₂ PO ₄ 10 mM Glucose 10 mM HEPES 1 mM MgCl ₂ (pH 7.4)

Table 2.3. cDNA constructs.

Construct Name	cDNA	Supplier	Reference
<i>GRKs</i>			
GRK5	pRK5-GRK5	Dr Robert Lefkowitz, HHMI/ Duke University Medical Centre, US.	(Pitcher et al., 1996)
GRK2	pRK5-GRK2	Dr Robert Lefkowitz	(Pitcher et al., 1999)
GRK3	pcDNA1-GRK3	Dr Robert Lefkowitz	(Menard et al., 1996)
GRK6A, B, C	pBK(Δ)-GRK6A, B, C	Dr Mario Tiberi, Universite de Montreal, Canada.	(Premont et al., 1999)
GST-CT	pEGB- β ARK-CT	Dr Robert Lefkowitz	(Pierce et al., 2001)
GRK2-K220R	pBC12B1- β ARK1-K220R	Dr Robert Lefkowitz	(Freedman et al., 1995)
GRK2-NT	pEGFP-GRK2-(45-178)-GFP	Dr Rachel Sterne-Marr	(Sterne-Marr et al., 2003)
Flag-GRK2 Flag-CAT	pcDNA3-GRK2-Flag pcDNA3-GRK2-185-543-Flag	Prof. Lan Ma	(Jiang et al., 2009)
GRK2-D110A	pcDNA3-GRK2-D110A	Dr Rachel Sterne-Marr	(Dhami et al., 2004)
GRK2-S/A	pcDNA3-S670A	Dr Robert Lefkowitz	(Pitcher et al., 1999)

GRK2-S/D	pcDNA3-S670D	Dr Robert Lefkowitz	(Pitcher et al., 1999)
GRK2-Y/F GRK2-Y/D	pcDNA3-Y13,86,92F pcDNA3-Y86,92F	Dr. Federico Mayor Jr, Universidad Autonoma Madrid	(Penela et al., 2001)
GRK2-W643A	pRK5-bovGRK2-W643A	Dr Robert Lefkowitz	(Touhara, 1998)
<i>Small GTPases</i>			
HA-Rho A*, B*, C*	pcDNA3.1-HA-RhoA (G14V) pcDNA3.1-HA-RhoB (G14V) pcDNA3.1-HA-RhoC (G14V)	Missouri S + T cDNA Resource Centre.	www.cdna.org
GST GST-RhoA-V14	pGEX6p1-EV pGEX6p1-RhoA-V14	Dr. Stephen Nurrish	(McMullan et al., 2006)
RhoA-WT RhoA-V14 RhoA-N19 RhoA-F25N RhoA-V14/F25N	pRK5-MYC-RhoA-WT pRK5-MYC-RhoA-V14 pRK5-MYC-RhoA-N19 pRK5-MYC-RhoA-F25N pRK5-MYC-RhoA-V14/F25N	Dr. Stephen Nurrish, LMCB, UCL	(McMullan et al., 2006)
Arf6-Q67L	pRK5-myc-Arf6 (Q67L)	Dr. Alan Hall	(Ridley et al., 1995)
Rac-Q61L	pRK5-myc-Rac (Q61L)	Dr. Alan Hall	(Nobes and Hall, 1995)
Cdc42-Q61L	pRK5-myc-Cdc42 (Q61L)	Dr. Alan Hall	(Nobes and Hall, 1995)
Ras-V12	pRK5-myc-Ras (G12V)	Dr. Alan Hall	(Ridley et al., 1995)

PTD-C3	pGEX-KG-PTD-Myc-C3	Prof. M. Olson	(Sahai and Olson, 2006)
Gq	pcDNA3-GαqQ209L-EE	Missouri S + T cDNA Resource Centre.	www.cdna.org
<i>MAP kinases</i>			
MEK-KA MEK-AA MEK-DD	pcDNA3-MEK1-K97A pcDNA3-MEK1-SA pcDNA1-MEK1-DD	Dr Robert Lefkowitz	(Lin et al., 1999)
ERK2	pEGFP-C1-ERK2	Dr. Rony Seger	(Rubinfeld et al., 1999)
MEK1 Raf-1	pRK5-myc-MEK-1 pEGFP-Raf-1	Dr. Pablo Rodriguez-Viciano, UCL Cancer Institute	(Rodriguez-Viciano et al., 2006)
<i>Others</i>			
AT1R	pcDNA3.1-HA-AT1AR	Dr. Marc Caron	(Zhang et al., 1996)
GIT	pBK(Δ)GIT1-Flag	Dr Robert Lefkowitz	(Premont et al., 1998)
PI3K	pcDNA3-PI3K	Dr. Phillip Hawkins	(Stephens et al., 1997)

3. RhoA_{GTP} is a novel binding partner for GRK2

3.1. GRK2 interacts specifically with active RhoA.

The GRKs are classically known for their ability to phosphorylate agonist occupied GPCRs. This is vital for switching off G protein-mediated signalling, a process known as desensitisation. The GRKs are now known to have a more complex role in regulating cell signalling mediated by their phosphorylation of non-receptor substrates and interactions with non-receptor binding partners. Thus, they can regulate various signalling pathways downstream of different classes of receptors in both positive and negative ways (discussed in detail in chapter 1). Using the 'BLOCKS' online sequence analysis tool, I identified regions homologous to HR1 Rho-binding repeats within the catalytic domain of GRK2. These Rho-binding repeats are found in Rho effectors such as rhophilin, rhotekin and PKNs 1-3. Figure 3.1A shows a ClustalW sequence alignment of the HR1a domain from PKN1 aligned with the HR1 domain from rhotekin and three different regions from the catalytic domain of GRK2 identified as having HR1 homology. GRK2 region 271-325 has the highest Blocks score for homology to HR1 (Block E-value of 6.2×10^{-15}) and this region also displays homology (Block E-value of 2.9×10^{-7}) to the PAK-box/p21-rho-binding domain found in Rho-binding proteins such as the serine/threonine kinase PAK1 (Figure 3.1B). The Block E-value represents the probability that the domain could be aligned by chance with the query sequence (GRK2) at that position (<http://blocks.fhcrc.org/blocks/help/help.html>). As a comparison, the Block E-value for the alignment of an RGS domain with the known RGS homology (RH) domain in the GRK2 amino-terminal region is 1.4×10^{-12} . I

therefore set out to test whether the small GTPase Rho may be a novel binding partner for GRK2.

(A)

PKN1_HR1a
Rhotekin_HR1
GRK2_271-325

LRREIRKELKKEGAENLRRATTDLGRSLGPVELLLRGSSRRLLDHHQQLQELH
LQRLDHEIRMRGACKLLAACSQREQALEATKLLVCNSRILSYMG-ELQRRK
LLDMNGGDLHYHLSQHGVFSEADMRFYAAEIIILGLEHMHNFVYRDLKPANIL

PKN1_HR1a
Rhotekin_HR1
GRK2_409-461

LRREIRKELK--LKEAENLRRA^{TD}LGRSLGPV^{ELL}RGSSRR^{LLD}LLHQQLQELH
LQRLKDHEIR--MREGACKLLAACSQR^{EAL}EAT^{KS}LLVCNSRILSYMG-ELQRRK
MAVELPDSFPELRSLL^{EGL}LQR--DVNRRRLGCLGRGAQ-EVKESPF^{FR}-SLDWQM

PKN1_HR1a
Rhotekin_HR1
GRK2_188-244

LRREIRKELKLKEGAENLRRATTDLGRSLGPVELLLRGSSRRLDLLHQQLQELH
LQRKLDHEIRMREGACKLLAACSQREQALEATKSLLVCNSRIILSYMG-ELQRRK
MN-DFSVHRIIGRGGFGEVYGCRA DTG--KMYAMKCLDK-----

(B)

PAK1_PAK-box/p21-RBD
GRK2 277-323

EISLPD FEHTIHVGFD AVTG EFTGMPEQWARLLQTSNITKSEQKNPQAVLDVLEFYN-
 ---GDLHYHLSQ---HGVFS---EADMRFYAAEIIIGLEHMHNRFFVYRDLPANI

Figure 3.1. ClustalW sequence alignment of HR1 domains from PKN1 and rhotekin (A) and PAK-box rho-binding domain from PAK1 (B) with regions of GRK2 catalytic domain. ‘*’ represents identical residue, ‘:’ represents strongly conservative substitution, ‘.’ represents weakly conservative substitution. Residues coloured blue are acidic, magenta are basic, red are hydrophobic and green are hydroxyl/sulphydryl/amine.

To test for a putative interaction between GRK2 and Rho I performed co-immunoprecipitation experiments in COS cells, which I used because they are easy to maintain and transfect. I co-expressed GRK2 with Myc-tagged constructs of two RhoA mutants: RhoA-V14 and RhoA-N19. RhoA, like most small GTPases, cycles between an active GTP-bound form and an inactive GDP-bound form, a process that is tightly regulated by GAPs and GEFs (section 1.4). RhoA-V14 is a mutant that lacks GTPase activity and thus constitutively mimics the active, GTP-bound form of RhoA while RhoA-N19 is a mutant that can’t bind to GTP and thus mimics the inactive, GDP-bound form of RhoA. The Rho constructs are tagged at the amino-terminus rather than the carboxyl-terminus so as not to interfere with vital carboxyl-terminal post-translational modifications (Robertson et al., 1995). Using an antibody against the Myc tag, I immunoprecipitated the small GTPases and found that GRK2 can be co-immunoprecipitated specifically with RhoA-V14 but not with RhoA-N19 (Figure 3.2A). Furthermore, GRK2 failed to co-immunoprecipitate with Myc-tagged, constitutively active mutants of other small GTPases Arf6-Q67L, Rac-Q61L, Ras-V12 or Cdc42-Q61L (Figure 3.2A). These results indicate that GRK2 interacts specifically with the active, GTP-bound form of RhoA. GRK2 is known to interact with Arf6 indirectly via GIT1 (Premont et al., 1998). That GRK2 co-immunoprecipitates with RhoA-V14, but fails to do so with Arf6-Q67L, and that it

contains regions homologous to Rho-binding repeats found in known Rho effectors, may indicate that the interaction between GRK2 and Rho_{GTP} is direct (section 3.3).

The family of Rho GTPases comprises 20 members, most of which have been shown to have roles in modulating the dynamics of the actin cytoskeleton. The family includes, among others, the Cdc42 and Rac subfamilies, which regulate filopodia and lamellipodia formation respectively. RhoA is a member of the classical RhoABC subfamily consisting of the highly conserved (85% amino acid sequence identity) RhoA, RhoB and RhoC, which are best known for their function in regulating stress fibre formation. The data in Figure 3.2A shows that GRK2 fails to interact with constitutively active mutants of the Rho family members Cdc42 and Rac as well as other small GTPases Ras and Arf6. However, co-immunoprecipitation experiments with HA-tagged constitutively active mutants of RhoA, RhoB and RhoC indicate that GRK2 binding is common to these close homologues of RhoA in the RhoABC subfamily (Figure 3.2B). This is possibly unsurprising as, despite different cellular functions of RhoA, B and C, known Rho effectors, including PKN, commonly bind to all three Rho isoforms (Wheeler and Ridley, 2004).

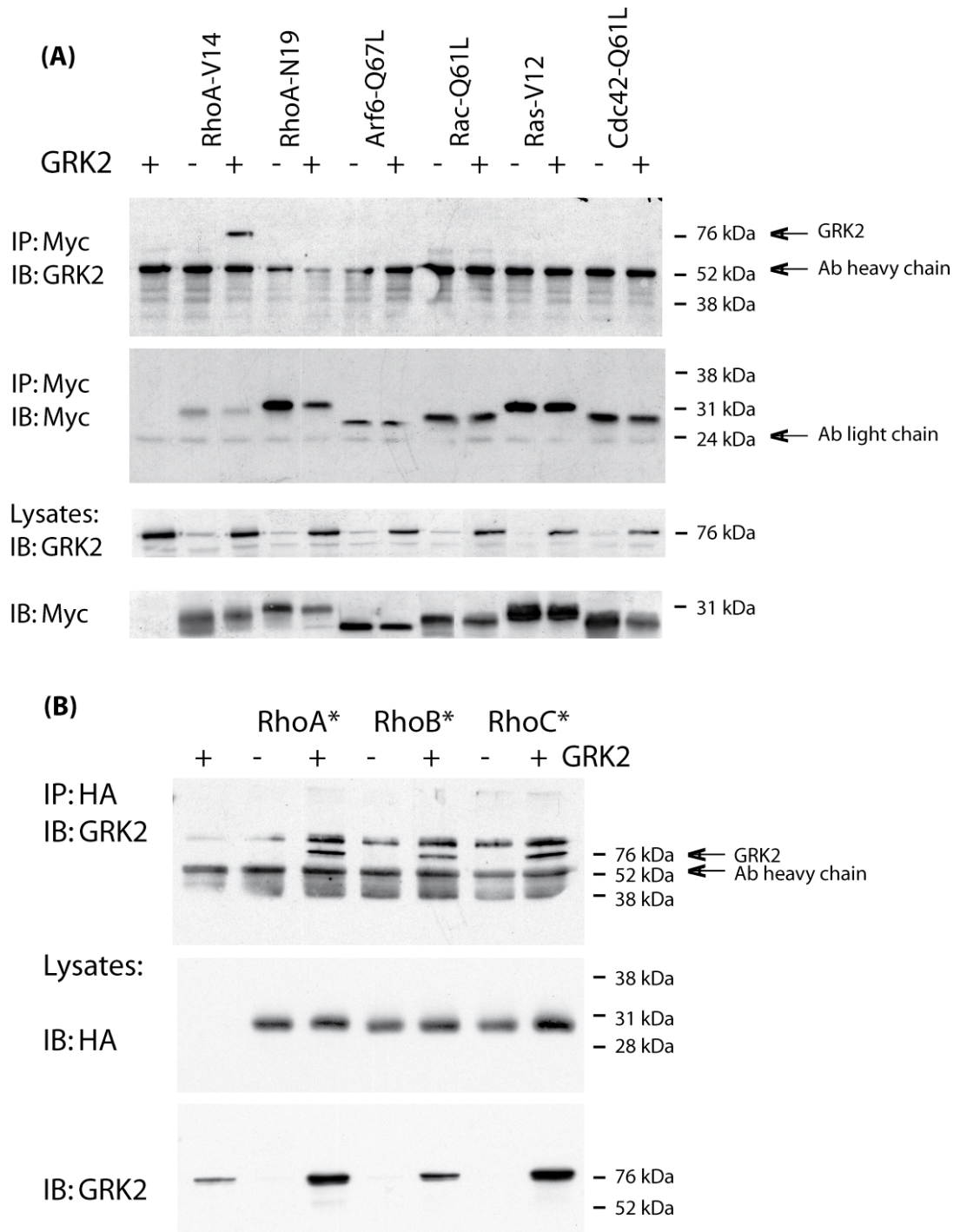


Figure 3.2. GRK2 interacts specifically with active RhoA_{GTP}. (A) COS cells were transfected as indicated, see table 2.3 for construct details. Protein expression was confirmed by western blotting (lysates) with expression of small GTPases confirmed using an anti-Myc antibody (rabbit). N-terminally Myc-tagged small GTPases were immunoprecipitated (IP) using an anti-Myc antibody (rabbit) and immunoprecipitates were probed (IB) with anti-GRK2-3 or anti-Myc (mouse) antibodies to test the IP efficiency. (B) COS cells were transfected as indicated and protein expression

confirmed by western blotting (lysates) with Rho expression confirmed using an anti-HA antibody. N-terminally HA-tagged constitutively active Rho constructs were immunoprecipitated (IP) using an anti-HA antibody and immunoprecipitates were probed (IB) for GRK2 content using an anti-GRK2-3 antibody. Western blots are representative of 3 separate experiments.

3.2. RhoA_{GTP} binding is a common feature of the GRKs.

The central catalytic domain of GRK2 (where the putative Rho-binding repeats are found) is highly conserved within the GRK family, suggesting that the ability to bind to RhoA_{GTP} might be common to the other GRKs. There are 7 GRKs in mammals, which are divided into 3 subfamilies based on sequence comparisons. GRKs 1 and 7 are only expressed in the retina while GRKs 2 and 3 of the GRK2 subfamily and GRKs 5 and 6 of the GRK5 subfamily are widely expressed. GRK4 is found at significant levels only in the kidney, testis and cerebellum (section 1.1). GRK6 is found in three splice variants, GRK 6A, 6B and 6C. I decided to perform further co-immunoprecipitation experiments with GRKs 2 and 3 of the GRK2 subfamily as these are 93% identical within their catalytic domains. I also tested for RhoA-V14 binding to GRKs 5, 6A, 6B and 6C of the GRK5 subfamily as these are also ubiquitously expressed and are the next closest relatives of GRK2 in terms of their catalytic domain sequences (49% and 51% sequence identity). Figure 3.3 shows that they are all able to co-immunoprecipitate with Myc-tagged RhoA-V14 when over-expressed in COS cells, suggesting that RhoA_{GTP} binding may be a common feature of the GRKs.

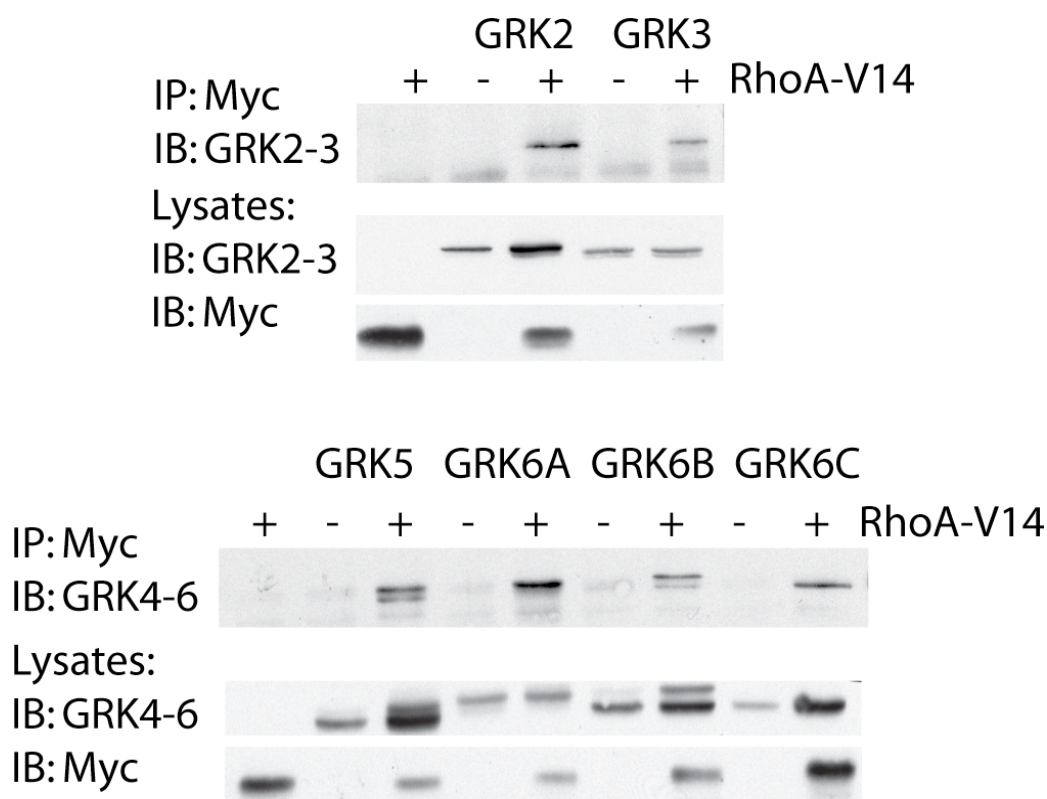


Figure 3.3. Rho binding is a common feature of the GRKs. COS cells were transfected as indicated. Protein expression was confirmed by western blotting (lysates) with expression of Myc-RhoA-V14 confirmed using an anti-Myc antibody (rabbit). Myc-RhoA-V14 was immunoprecipitated (IP) using an anti-Myc antibody (rabbit) and immunoprecipitates were probed (IB) with anti-GRK2-3 or anti-GRK4-6 antibodies. Western blots are representative of 3 separate experiments.

The fact that RhoA_{GTP} binding is a common feature of GRKs 2, 3, 5 and 6, together with the observation that the GRK2 catalytic domain contains regions homologous to Rho-binding domains suggest that GRK2 binds to Rho_{GTP} via its catalytic domain. In order to confirm which domain of GRK2 is responsible for Rho binding, I co-expressed tagged constructs of the amino-terminal domain, amino acids 45-178 (GFP-NT), catalytic domain 185-543 (Flg-CAT) and carboxyl-terminal domain 492-689 (GST-CT) of GRK2 with Myc-tagged RhoA-V14 in COS cells. The catalytic domain exhibits the strongest interaction with RhoA-V14, as shown by

co-immunoprecipitation (Figure 3.4A), confirming that GRK2 binds to Rho_{GTP} via its catalytic domain. Quantifying the amount of GRK2 co-immunoprecipitated per unit RhoA-V14 immunoprecipitated reveals that the catalytic domain of GRK2 binds approximately three times better than the full-length GRK2 construct suggesting that, *in vivo*, a conformational change in GRK2 may be required to facilitate Rho_{GTP} binding (Figure 3.4B). Interestingly, while the interaction with the carboxyl terminus (CT) of GRK2 was poor, the interaction with the amino terminus (NT) was as strong as with full-length GRK2, indicating that the amino-terminal domain of GRK2 may also contribute to RhoA-V14 binding (Figure 3.4A and 3.4B).

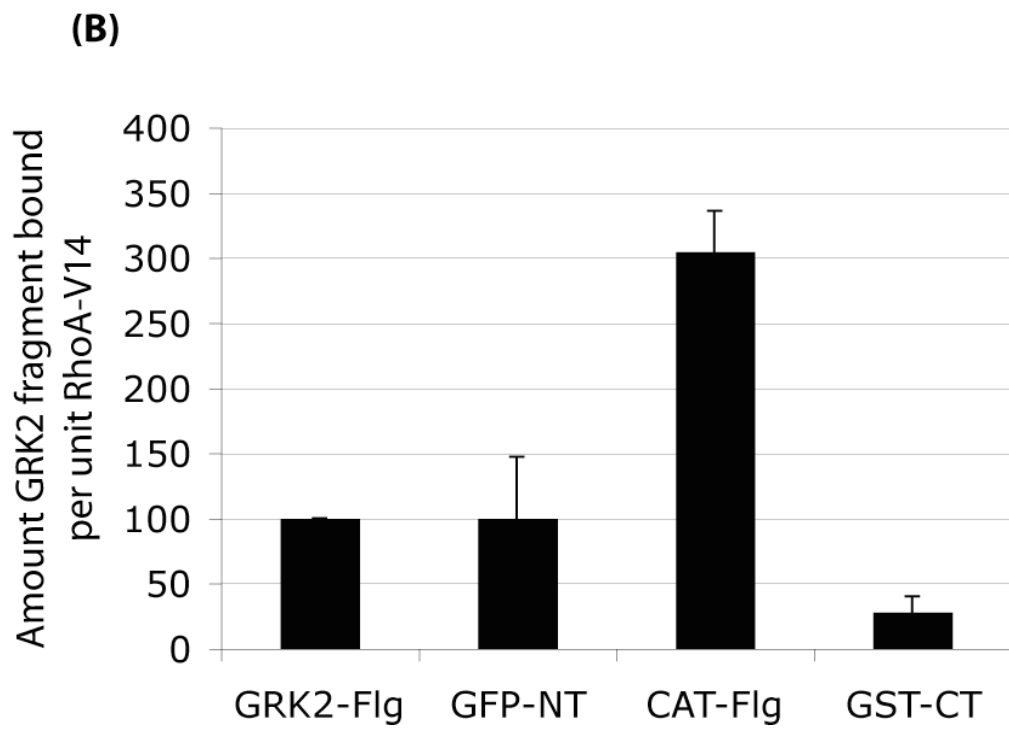
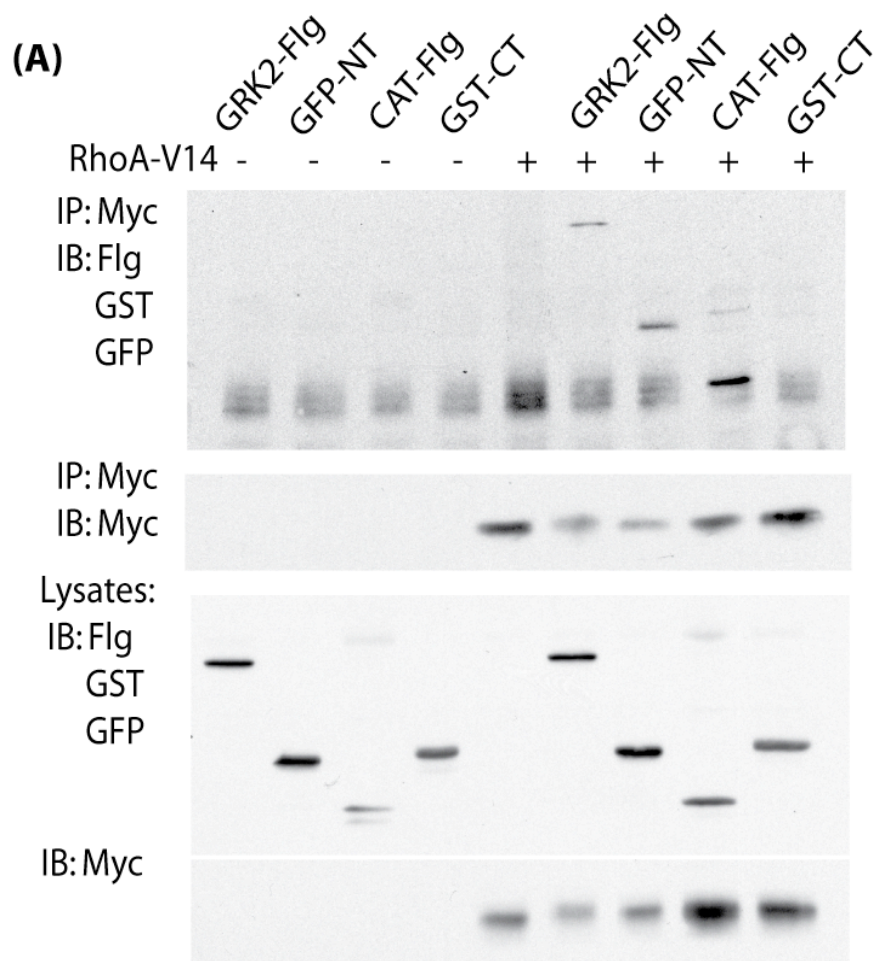


Figure 3.4. GRK2 interacts with active RhoA via its catalytic domain. (A) COS cells were transfected as indicated and protein expression confirmed by western blotting (lysates). Myc-tagged RhoA-V14 was immunoprecipitated (IP) using an anti-Myc antibody (rabbit) and immunoprecipitates were probed (IB) with anti-Flg, GST and GFP antibodies or with an anti-Myc antibody (mouse) to confirm efficiency of IP. The western blots shown are representative of 3 separate experiments. (B) Quantification of (A), amount of GRK2 fragment co-immunoprecipitated per unit RhoA-V14 immunoprecipitated is plotted relative to amount of co-immunoprecipitation seen for full length GRK2, error bars represent standard deviation from the mean of 3 separate experiments.

3.3. GRK2 interacts with RhoA_{GTP} directly but fails to interact with the RhoA mutant F25N.

Although suggestive, co-immunoprecipitation of two proteins does not conclusively indicate that they interact directly with each other. It is possible that other proteins within the cell lysate are required to mediate the interaction. To test whether GRK2 interacts directly with RhoA_{GTP}, I performed GST pull-down experiments with a GST-RhoA-V14 fusion protein (or a GST negative control) purified from bacterial lysates. These purified fusion proteins attached to glutathione-sepharose beads were incubated with *in vitro*-translated [³⁵S]-methionine-labelled GRK2 or GRK2 catalytic domain as described in chapter 2. After washing, bound protein was removed from the beads and samples run on SDS-PAGE gels. The gels were dried and exposed to film. Figure 3.5 shows that full-length *in vitro*-translated GRK2 and GRK2 catalytic domain can be pulled down with GST-RhoA-V14 but not with GST, indicating that GRK2 interacts directly with RhoA-V14, at least in part, via its catalytic domain.

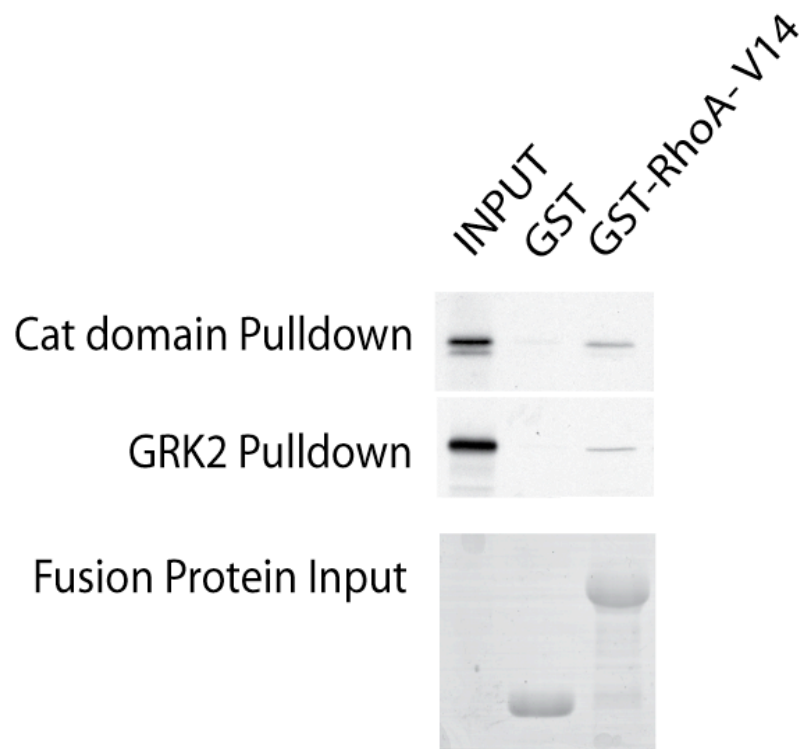


Figure 3.5. GRK2 interacts directly with active RhoA-V14. GST or GST-RhoA-V14 was used to pull down *in-vitro* translated GRK2 or GRK2 catalytic domain. Samples were run on SDS-PAGE gels alongside 1 ul of *in-vitro* translated GRK2 or GRK2 catalytic domain input and gels were dried and exposed to film. The fusion protein inputs were checked by running equal amounts of GST and GST-RhoA-V14 on SDS-PAGE gels before coomassie staining. Representative of 3 separate experiments.

The ability of GRK2 to interact specifically and directly with RhoA_{GTP} may indicate that it is a novel Rho effector. The RhoA mutant RhoA-V14/F25N is a stabilised mutant of RhoA that was originally designed to aid in its purification. It happens that it binds normally to most of its effectors, including PKN, mDia and ROCK but is impaired in its binding to some other effectors such as diacylglycerol (DAG) kinase θ (DGK θ) and therefore may be a useful tool in categorising RhoA effectors (McMullan et al., 2006). I have found that GRK2 binds poorly to the RhoA

mutant RhoA-V14/F25N (Figure 3.6) suggesting that GRK2 may belong to the ‘DGKθ class’ of Rho effectors.

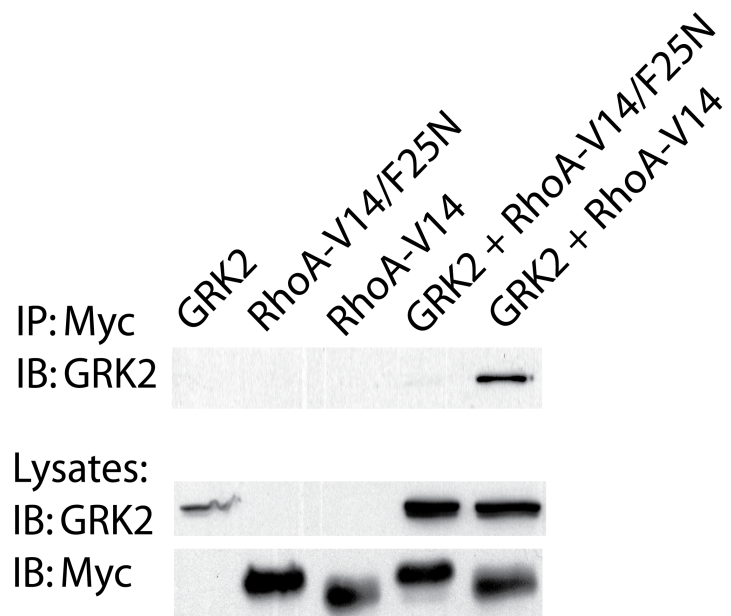


Figure 3.6. GRK2 fails to interact with RhoA-V14/F25N. COS cells were transfected as indicated and protein expression was confirmed by western blotting (lysates) with Rho constructs detected using an anti-Myc antibody. Myc-tagged Rho constructs were immunoprecipitated (IP) using an anti-Myc antibody (mouse) and immunoprecipitates were probed (IB) with an anti-GRK2 antibody (rabbit). Western blots are representative of 3 separate experiments.

3.4. Summary.

In summary, the data in this chapter identifies RhoA_{GTP} as a novel binding partner for GRK2. The interaction is specific and direct and mediated predominantly by the GRK2 catalytic domain. Rho binding is common to all of the ubiquitously expressed GRKs. While GRK2 binds equally well to RhoA, B and C, it fails to interact with the RhoA mutant F25N, suggesting that GRK2 may belong to the emerging ‘DGKθ class’ of Rho effectors. The fact that the GRK2 catalytic domain in isolation interacts with RhoA-V14 three times more strongly than full length GRK2 suggests that, *in vivo*, a conformational change in GRK2 may be required to expose the GRK2 catalytic domain for full RhoA_{GTP} binding. The additional observation that the amino-terminal region of GRK2 interacts with RhoA-V14 equally as well as full length GRK2 indicates that the amino-terminal region of GRK2 also contributes to RhoA_{GTP} binding.

It is tempting to speculate that initial binding of RhoA_{GTP} to the amino-terminus of GRK2 may promote a conformational change in GRK2 to expose the GRK2 catalytic domain for full RhoA_{GTP} binding. A modification to full length GRK2, such as phosphorylation, might also be required to expose the GRK2 catalytic domain *in vivo*. The lack of such a modification to GRK2 in the co-immunoprecipitation experiments could explain why the full length construct cannot bind to RhoA-V14 as effectively as the catalytic domain alone. GRK2 phosphorylation may directly affect a conformational change in GRK2 or alternatively could increase RhoA_{GTP} binding in the amino-terminal region of GRK2, which would then in turn act to expose the catalytic domain for full RhoA_{GTP}

binding. I next wanted to investigate whether RhoA_{GTP} binding to GRK2 affects the cellular functions of GRK2.

4. RhoA_{GTP} binding to GRK2 promotes GRK2/Raf1, GRK2/MEK1 and GRK2/ERK2 complex formation

4.1. GRK2-mediated GPCR desensitisation and GRK2 kinase activity are unaffected by binding to RhoA_{GTP}.

In chapter 3 I demonstrated that RhoA_{GTP} is a novel binding partner for GRK2. I observed that RhoA_{GTP} binding is predominantly mediated by the catalytic domain of GRK2 and hence I hypothesised that Rho might affect the enzymatic activity of the kinase. GRK2 classically phosphorylates GPCRs resulting in their desensitisation (section 1.1.2) and so I set out to test whether RhoA_{GTP} binding to GRK2 affects its ability to desensitise GPCRs. I decided to look at the Angiotensin_{II} type 1A receptor (AT1R) as GRK2 has been shown to desensitise AT1R-dependent signalling both in cells (Kim et al., 2005) and *in vivo* (Eckhart et al., 2002). AT1R is coupled to the heterotrimeric G protein Gq, which activates PLC β . PLC β cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) into the second messengers DAG and IP₃, which causes, among other downstream effects, release of calcium from the endoplasmic reticulum. I assayed for AT1R-mediated Gq activation in COS cells by measuring IP₃ production in response to ANG_{II} as described in chapter 2.

COS cells transfected with AT1R display a 6.7 ± 0.7 fold increase in IP₃ production over basal levels when treated for 30 min with ANG_{II} (Figure 4.1A). When the cells are co-transfected with GRK2, the response to ANG_{II} is desensitised by $72 \pm 4\%$ relative to cells expressing the receptor alone. GRK2 has been shown to desensitise Gq-coupled receptors using the classical phosphorylation-dependent

mechanism but also by directly binding and sequestering Gq using its RH domain (section 1.1.2). Sequestration of Gq by the RH domain of GRK2 prevents reactivation of the heterotrimeric G protein by the receptor and inhibits Gq_{GTP} interaction with its effectors. GRK2-K220R is a mutant of GRK2 that is devoid of kinase activity. K220R can thus only desensitise Gq signalling by kinase-independent mechanisms, such as by binding and sequestering Gq via its RH domain. GRK2-D110A is a mutant of GRK2 that is unable to bind and sequester Gq and as such is predicted to desensitise Gq signalling by phosphorylating the receptor. IP₃ production in response to ANG_{II} in COS cells is also desensitised by co-expression of either K220R (90 ± 2% desensitisation) or D110A (62 ± 4%) with AT1R, demonstrating that in this system GRK2 desensitises AT1R by phosphorylation-dependent and independent mechanisms (Figure 4.1A and B). Note that I was unable to detect receptor expression by western blot and therefore cannot be sure that reduction in receptor expression may in part account for the reduction in IP₃ production upon GRK2 transfection. In future, I could perform radioligand binding experiments to ensure that the cell surface levels of receptor are consistent across experimental conditions.

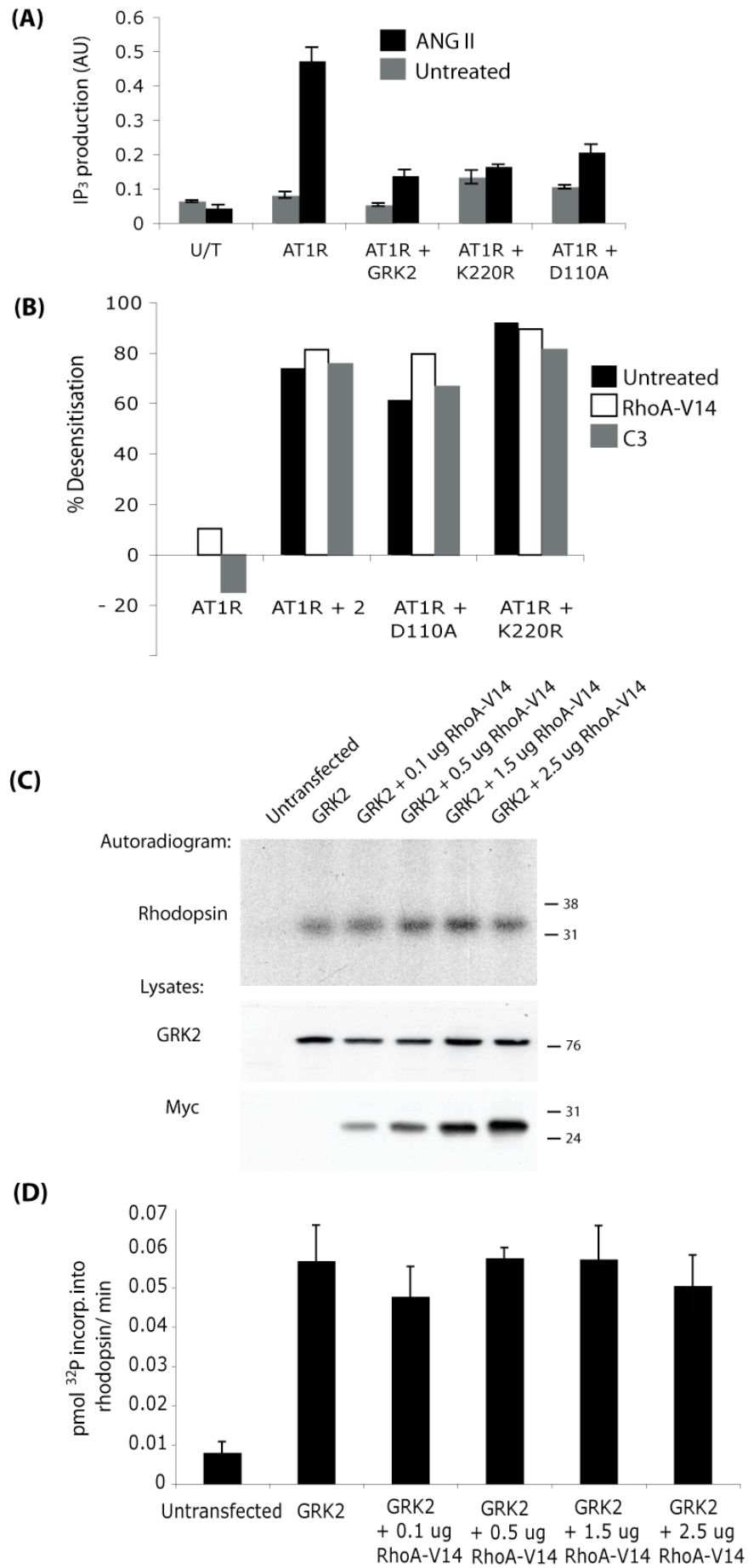


Figure 4.1. GRK2-mediated GPCR desensitisation and GRK2 kinase activity are unaffected by Rho. Cos cells were transfected as indicated. **(A)** IP₃ production was measured as amount of labeled inositol incorporated into IP₃ per amount incorporated into total lipid as described in chapter 2. Dark grey bars represent cells treated with 1 μ M ANG_{II} for 30 min. Error bars represent standard deviation from mean of 3 separate experiments. **(B)** % desensitisation of IP₃ production was calculated as the difference in IP₃ production in response to ANG_{II} in the presence versus the absence of GRK2, divided by IP₃ production in the absence of GRK2. Empty bars represent cells co-transfected with RhoA-V14, light grey bars represent cells pre-incubated overnight with 0.5 μ M C3. A representative is shown from experiments performed 2-3 times. **(C and D)** Cell lysates mock transfected or expressing GRK2 +/- increasing amounts of RhoA-V14 cDNA were incubated with Rod Outer Segments (ROS) and GRK2 kinase activity was measured as pmol of labeled Pi incorporated into rhodopsin/ min as described in chapter 2. (C) shows a representative autoradiogram and western blots to demonstrate GRK2 and Rho (Myc) expression in the lysates. In (D), autoradiograms are quantified using QuantityOne (BioRad) and error bars represent standard deviation from the mean of 4 separate experiments.

Next, to assess whether Rho affects phosphorylation-dependent or independent desensitisation of AT1R-mediated Gq signalling by GRK2, I performed the same experiments but in the presence of co-expressed RhoA-V14 or with pre-incubation overnight with the RhoABC inhibitor C3. Figure 4.1B shows that neither the presence of exogenous constitutively active RhoA-V14, nor the presence of the Rho inhibitor C3 affect the ability of wildtype, K220R or D110A GRK2 to desensitise IP₃ production in response to ANG_{II}. Thus, Rho does not appear to affect phosphorylation-dependent or independent desensitisation of AT1R-mediated Gq signalling by GRK2.

In agreement with these findings, *in vitro* kinase assays demonstrated that GRK2 kinase activity is unaffected by RhoA_{GTP}. I assessed the ability of COS cell lysates over-expressing GRK2 to phosphorylate the GRK2 substrate rhodopsin *in*

vitro as described in chapter 2. Lysates with over-expressed GRK2 displayed an 8.1 ± 1.2 fold increase in the rate of rhodopsin phosphorylation relative to untransfected control lysates. Co-expression of increasing amounts of RhoA-V14 in the lysates did not significantly affect the ability of over-expressed GRK2 to phosphorylate rhodopsin, indicating that the kinase activity of GRK2 is unaffected by RhoA_{GTP} binding (Figure 4.1C and D).

The *in vitro* kinase assays performed in Figure 4.1C also suggested that RhoA is not a substrate for GRK2. The [³²P]-labelled proteins (visualised by the BioRad molecular imager FX) displayed a strong band at the molecular weight of rhodopsin indicating the incorporation of radiolabelled phosphate into the known GRK2 substrate. There were however no bands at the molecular weight of RhoA (~25 kDa), indicating that no radiolabelled phosphate was incorporated into RhoA in the *in vitro* reaction (Figure 4.1C). Thus, it seems likely that, despite the fact that RhoA_{GTP} binds to the catalytic domain of GRK2, this binding does not affect GRK2 kinase activity or GPCR desensitisation, and there is no indication that RhoA itself is a substrate. A more detailed analysis of the kinetics of GRK2-mediated peptide (soluble substrate) phosphorylation in the presence or absence of RhoA-V14 is probably warranted. A comparison of GRK2-mediated RhoA phosphorylation with a known RhoA kinase, such as PKA (Tkachenko et al., 2011) would also provide further evidence that GRK2 is not a RhoA kinase.

4.2. RhoA_{GTP} binding to GRK2 promotes GRK2 binding to a subset of its non-receptor binding partners.

The GRKs are now known to have a more complex role in regulating cell signalling beyond their classical role in GPCR desensitisation, mediated by GRK2-dependent phosphorylation of, and/or interactions with, several non-receptor substrates and binding partners (sections 1.1.5 – 1.1.7). Given that I found no effect of RhoA_{GTP} binding to GRK2 on its classical role in GPCR desensitisation, I next wanted to test whether RhoA binding to GRK2 may affect any of the non-classical functions of GRK2. To do this, I performed further co-immunoprecipitation experiments to assess whether the interaction of GRK2 with any of its non-receptor binding partners is affected by RhoA_{GTP} binding. I first looked at binding of GRK2 to four of its known binding partners; phosphatidylinositol 3-kinase γ (PI3K γ); the MAPK kinase MEK1; the heterotrimeric G protein Gq and GIT1, a multi-functional protein identified as a GRK2 binder in a yeast 2-hybrid screen. The functions of the interactions of these proteins with GRK2 are discussed in detail in chapter 1.

When over-expressed in COS cells, I could detect an interaction of PI3K, Gq and GIT1, but not MEK1 with GRK2 (Figure 4.2A, B, C and D). Upon co-expression of RhoA-V14, the co-immunoprecipitation of GRK2 with PI3K γ , GIT1 and Gq was equivalent to that seen in the absence of RhoA-V14 expression, indicating that RhoA does not affect GRK2 binding to PI3K γ , GIT1 or Gq (Figure 4.2A, B and C). Incidentally, the equivalent binding of GRK2 to Gq in the presence or absence of RhoA-V14 is in agreement with data shown in Figure 4.1A and B. There I showed that phosphorylation-independent desensitisation of Gq signalling downstream of the AT1R by kinase-dead GRK2 is unaffected by the presence of

RhoA-V14. Given that this phosphorylation-independent desensitisation is mediated by direct binding of GRK2 to Gq, you might expect that co-immunoprecipitation of the two proteins should also be unaffected by RhoA-V14, and this is what I see in Figure 4.2C.

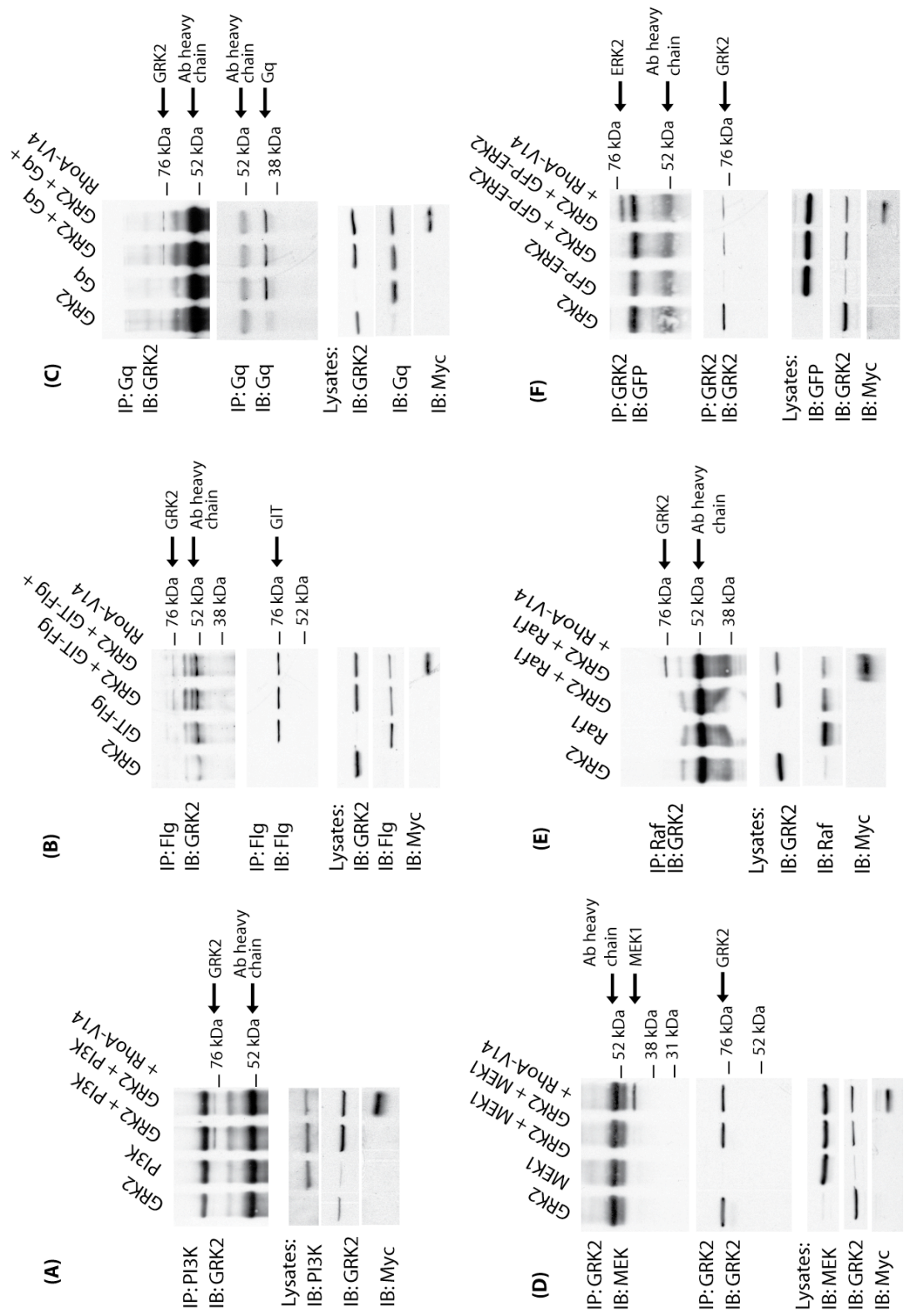


Figure 4.2. RhoA_{GTP} binding to GRK2 promotes GRK2 binding to Raf1, MEK1 and ERK2 but not to other binding partners, PI3K, Gq and GIT. COS cells were transfected as indicated and expression confirmed by western blotting (lysates) with expression of RhoA-V14 confirmed by blotting with an anti-Myc antibody (rabbit), GIT expression with an anti-Flg antibody and ERK2 with an anti-GFP antibody. The indicated proteins were immunoprecipitated (IP) and immunoprecipitates were probed (IB) with antibodies against the indicated proteins/epitopes. Western blots are representative of 3 separate experiments.

In marked contrast to the other binding partners, co-immunoprecipitation of GRK2 with MEK1 was strongly potentiated by co-expression of RhoA-V14 (Figure 4.2D). This data suggests that RhoA-V14 binding to GRK2 acts to promote GRK2 binding to MEK1 and was the first indication of a possible function for the interaction between GRK2 and RhoA_{GTP}.

The function of MEK is to phosphorylate the MAP kinase ERK in response to phosphorylation and activation by its upstream kinase Raf. This Raf/MEK/ERK MAPK cascade has many varied and important functions in cell biology including regulating expression of genes required for cell growth and proliferation as well as phosphorylation of a number of other nuclear and cytosolic substrates in response to many different stimuli. Scaffold proteins bind to two or more of the components of the cascade in order to promote their phosphorylation and hence regulate the strength, duration and/or sub-cellular localisation of ERK activation. This is important to regulate the specificity of cellular responses downstream of ERK (section 1.2). I therefore wanted to test whether GRK2 could also bind to the upstream MAPKKK Raf and/or the downstream MAPK ERK. I found that, as for MEK1, the co-immunoprecipitation of GRK2 with Raf1 and ERK2 is, in both cases, potentiated by co-expression of RhoA-V14 (Figure 4.2E and F). While MEK1

(Jimenez-Sainz et al., 2006) is a known binding partner for GRK2 and ERK1 has been reported to interact with GRK2 in response to activation of the β_2 AR (Elorza et al., 2000), the observed interactions with Raf1 and ERK2 have not previously been shown. Thus, RhoA-V14 binding to GRK2 specifically promotes GRK2 binding to all 3 components of the Raf/MEK/ERK MAP kinase cascade, suggesting that GRK2 may have a function as a Rho-activated ERK MAP kinase scaffold protein.

4.3. RhoA mutants RhoA-N19 and RhoA-V14/F25N fail to promote GRK2 binding to MEK1 and ERK2.

In section 3.1, I showed that GRK2 interacts specifically with the active mutant of RhoA, RhoA-V14, which mimics the GTP-bound form of the small GTPase but not with the inactive mutant, RhoA-N19, which mimics its GDP-bound form. Furthermore, in section 3.3, I found that GRK2 fails to interact with the RhoA-V14 mutant F25N, suggesting that GRK2 belongs to a select subset of Rho effectors. If GRK2 is indeed a Rho effector, whose function is to scaffold Raf1, MEK1 and ERK2 in response to RhoA_{GTP} binding, you would expect that expression of RhoA-N19 should fail to promote GRK2 binding to Raf1, MEK1 and ERK2 as a Rho effector should function only in response to active GTP-bound Rho. Similarly, the F25N mutant of RhoA-V14 should also fail to promote GRK2 binding to Raf1, MEK1 and ERK2 as, while this mutant does mimic the active form of RhoA and interacts normally with most of its effectors (McMullan et al., 2006), it does not bind to GRK2 (Figure 3.6). If the F25N mutant was to promote binding of GRK2 to Raf1, MEK1 and ERK2, it would suggest that Raf1, MEK1 and ERK2 binding was not a direct consequence of RhoA-V14 binding to GRK2 but rather an indirect

consequence of RhoA-V14 signalling in the cells, which is not consistent with a role for GRK2 as a Rho-effector.

Figure 4.3A shows that RhoA-V14, but not RhoA-N19, potentiates co-immunoprecipitation of GRK2 with MEK1, consistent with GRK2 being a Rho effector that binds MEK1 in response to RhoA_{GTP} binding (compare lane 'GRK2 + MEK + RhoA-V14' with lane 'GRK2 + MEK + RhoA-N19' in Figure 4.3A). Expression of wildtype RhoA with GRK2 and MEK1 results in a small potentiation of GRK2-MEK1 binding (GRK2 + MEK + RhoA-WT in Figure 4.3A). Presumably a proportion of the over-expressed wildtype RhoA is in its activated, GTP-bound form. Figure 4.3B demonstrates that GRK2 binding to MEK1 and ERK2 is a direct consequence of RhoA_{GTP} binding to GRK2 and not an indirect consequence of RhoA signalling, as evidenced by the fact that the F25N mutant of RhoA-V14, which fails to interact with GRK2, fails to promote co-immunoprecipitation of GRK2 with MEK1 (Figure 4.3B, top panel) or GFP-ERK2 (Figure 4.3B, second panel from top). Thus, further to the results in section 4.2, these data support a novel function for GRK2 as a Rho-activated scaffold protein for the ERK MAPK cascade.

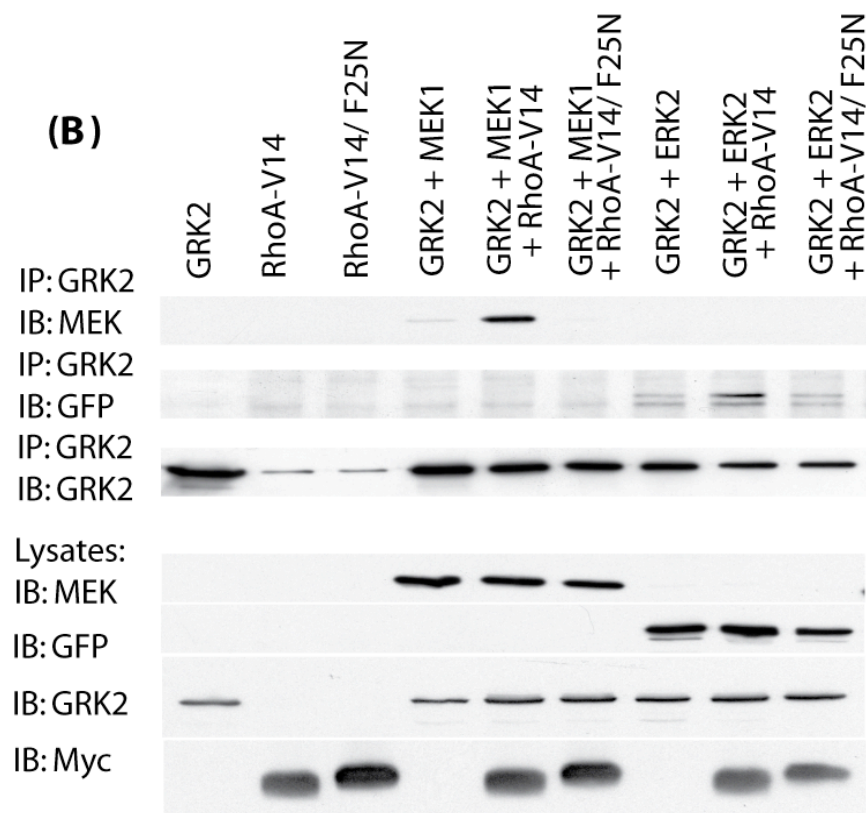
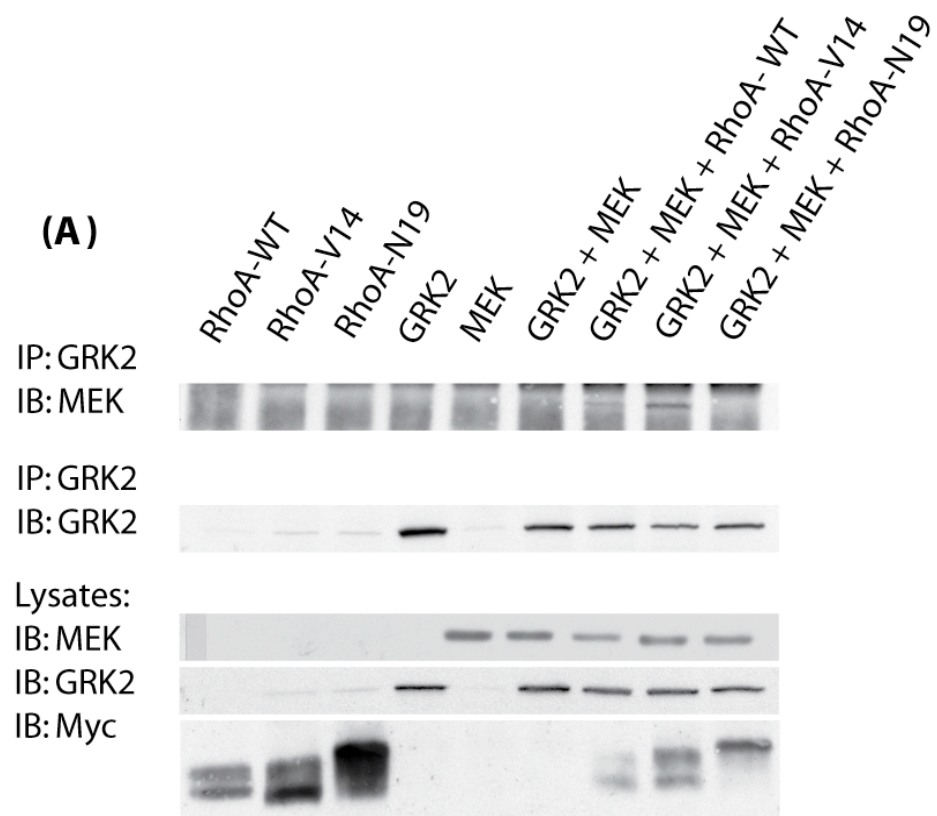


Figure 4.3. RhoA mutants RhoA-N19 and RhoA-V14/F25N fail to promote GRK2 binding to MEK1 and ERK2. Cos cells were transfected as indicated and protein expression was confirmed by western blotting (lysates) with Rho constructs detected using an anti-Myc antibody (mouse) and ERK2 with an anti-GFP antibody. GRK2 was immunoprecipitated (IP) using an anti-GRK2 antibody and immunoprecipitates were blotted (IB) with anti-MEK and GFP (ERK) antibodies or an anti-GRK2-3 antibody to check IP efficiency. Western blots are representative of 3 separate experiments.

4.4. The MEK1 binding site on GRK2 spans the catalytic and carboxyl terminal domains.

In order to determine which domain of GRK2 is responsible for MEK1 binding, I over-expressed tagged constructs of the amino-terminal domain, amino acids 45-178 (GFP-NT), catalytic domain 185-543 (Flg-CAT) and carboxyl-terminal domain 492-689 (GST-CT) of GRK2 with MEK1 in COS cells. As previously shown, full length GRK2 co-immunoprecipitates poorly with MEK1 unless RhoA-V14 is co-expressed (Figure 4.4A). In contrast, Flg-CAT and GST-CT both co-immunoprecipitate with MEK1 in the absence of RhoA-V14. GFP-NT, however, fails to interact with MEK1 (Figure 4.4A). Quantifying the amount of GRK2 immunoprecipitated per unit MEK1 suggests that there is no statistically significant difference between the ability of the catalytic domain and the carboxyl-terminal domain of GRK2 to interact with MEK1, as compared to full length GRK2 in the presence of RhoA-V14 (Figure 4.4B). The MEK1 binding site on GRK2 likely spans the catalytic and carboxyl-terminal domains and both domains may be required for full MEK1 binding. In full length GRK2, the MEK binding site is presumably occluded until RhoA-V14 binds. This implies that RhoA-V14 binding to

GRK2 causes a conformational change to expose a MEK1 binding site in the catalytic/carboxyl-terminal domain.

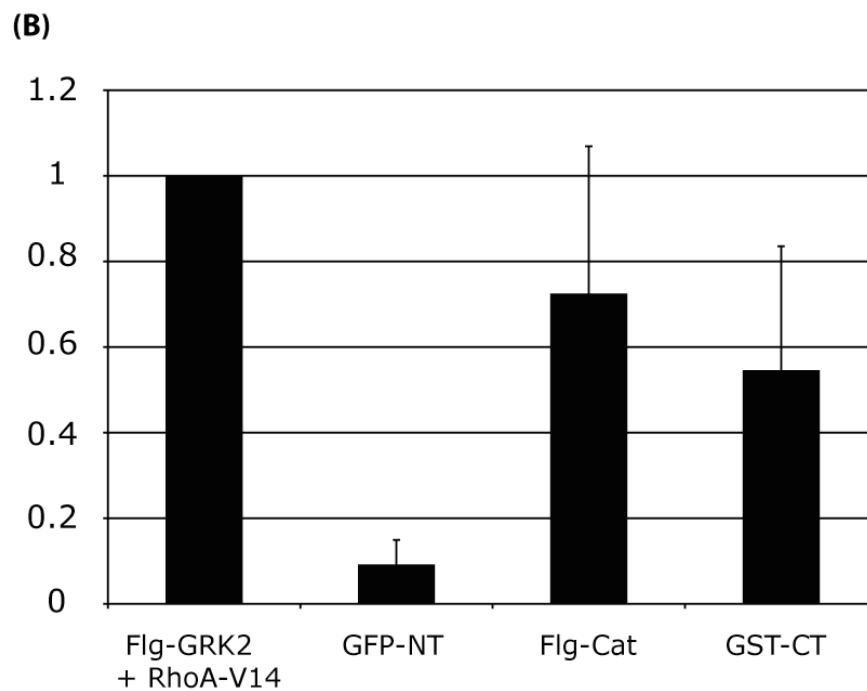
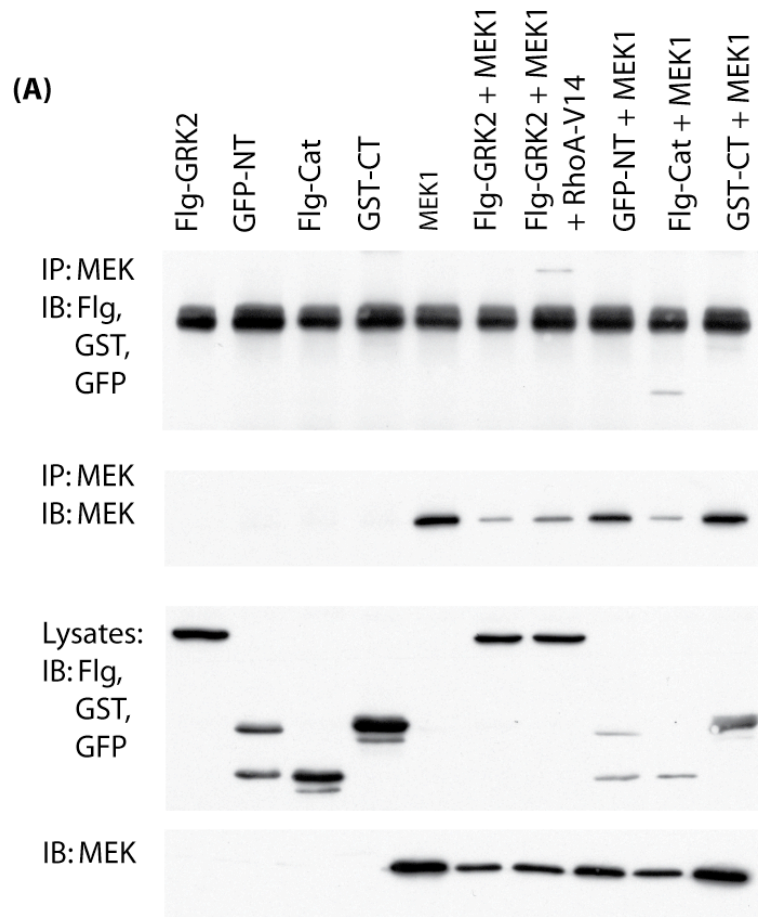


Figure 4.4. The MEK1 binding site on GRK2 spans the catalytic and carboxyl terminal domains. (A) COS cells were transfected as indicated and protein expression confirmed by western blotting (lysates). MEK1 was immunoprecipitated (IP) and immunoprecipitates were probed (IB) with a mixture of an anti-Flg antibody for detection of Flg-CAT, an anti-GST antibody for detection of GST-CT and an anti-GFP antibody for detection of GFP-NT or with an anti-MEK antibody to check IP efficiency, representative of 3 separate experiments. (B) Quantification of (A), amount of GRK2 fragment co-immunoprecipitated per unit MEK1 immunoprecipitated is plotted as arbitrary units relative to amount full length GRK2 co-immunoprecipitated, error bars represent standard deviation from the mean of 3 separate experiments.

4.5. GRK2-W643A, a PH-domain mutant of GRK2, interacts with MEK1 independently of RhoA-V14.

In order to gain further insight into the potential mechanisms whereby Raf/MEK/ERK scaffolding by GRK2 may be regulated, I decided to test the ability of various mutants of GRK2 to bind to MEK1 in the presence or absence of RhoA-V14. GRK2 can itself be phosphorylated by ERK at Serine residue 670, an event that has been shown to regulate enzymatic activity of the kinase (section 1.1.3). GRK2-S/A is a mutant of GRK2 with an alanine substitution at this phosphorylation site, preventing ERK phosphorylation, while GRK2-S/D is a mutant with an aspartate residue at this site, mimicking phosphorylation by ERK. Since I have mapped the MEK1 binding site partly to the carboxyl-terminal region of GRK2, I looked to see whether phosphorylation in this region altered MEK1 binding. GRK2-S/A and GRK2-S/D both co-immunoprecipitate with MEK1, following RhoA-V14 co-expression (Figure 4.5). Although the blots were quite variable, over three separate experiments, there was no clear or consistent difference in the ability of GRK2-WT, GRK2-S/A, GRK2-S/D, GRK2-K220R, and the Gq-binding deficient

mutant, GRK2-D110A to interact with MEK1, suggesting that binding is kinase activity- and Gq-independent and is unaffected by phosphorylation of S670 (Figure 4.5).

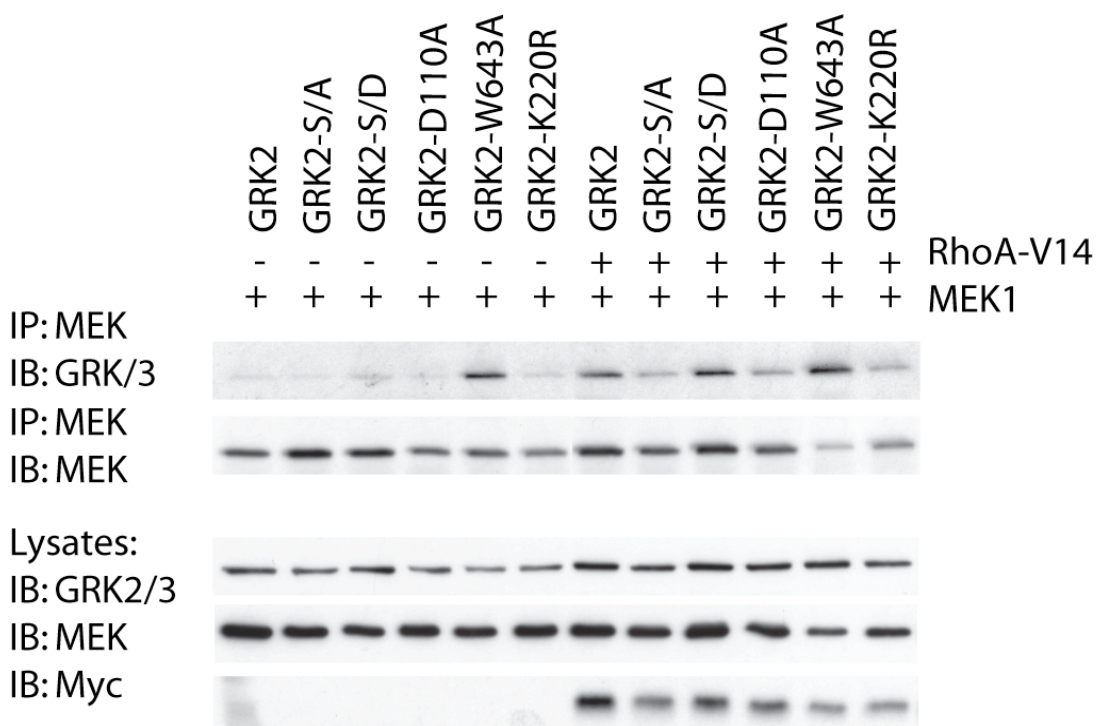


Figure 4.5. GRK2-W643A, a PH-domain mutant of GRK2, interacts with MEK1 independently of RhoA-V14. COS cells were transfected as indicated and protein expression confirmed by western blotting (lysates) with RhoA-V14 expression confirmed using an anti-Myc antibody. MEK1 was immunoprecipitated (IP) and immunoprecipitates were blotted (IB) with an anti-GRK2-3 antibody or with an anti-MEK antibody to check IP efficiency.

The GRK2-W643A mutant does however display an interesting and consistent difference to wildtype GRK2 as it co-immunoprecipitates equally well with MEK1 whether or not RhoA-V14 is co-expressed, indicating that this mutant of GRK2 interacts with MEK1 independently of Rho (Figure 4.5). GRK2-W643A is a point mutation in the GRK2 PH-domain that prevents its binding to G β γ (Touhara,

1998), presumably by causing a change in the conformation of the carboxyl-terminal region of the kinase. The W643A mutation may therefore serve to mimic the conformational change that is elicited by RhoA-V14 binding to wildtype GRK2 (discussed in the previous section), thus allowing binding to MEK1 in the absence of RhoA-V14. GRK2-W643A still, however, binds RhoA_{GTP} as well as wildtype GRK2 (data not shown). Overall, the binding of the GRK2 mutants to MEK was quite variable between experiments, the only consistent difference being the binding of GRK2-W643A to MEK in the absence of RhoA-V14 expression.

Further co-immunoprecipitation experiments also showed that the Rho-dependent interaction of GRK2 with MEK1 is independent of the activation state of MEK1. MEK1-KA has an alanine substitution at lysine residue 97 and MEK1-AA has a double alanine substitution at serine residues 218 and 222. Both of these mutations inhibit MEK1 kinase activity and act as dominant negatives. MEK1-DD is a constitutively active mutant of MEK1, which can phosphorylate ERK2 irrespective of its own phosphorylation state. All three of these mutants co-immunoprecipitate with GRK2 in a RhoA-V14-dependent fashion (Figure 4.6). While there may be slight variations in the amount of each mutant that co-immunoprecipitates with GRK2, the fact that none of them fail to interact supports a Raf/MEK/ERK scaffolding role for GRK2. If MEK1 or GRK2 were substrates for one another, you might expect their interaction to depend on MEK1 activation state. In contrast, a scaffold protein functions by binding to and facilitating the activation of MEK1 and hence would be predicted to bind to MEK1 regardless of its activation state.

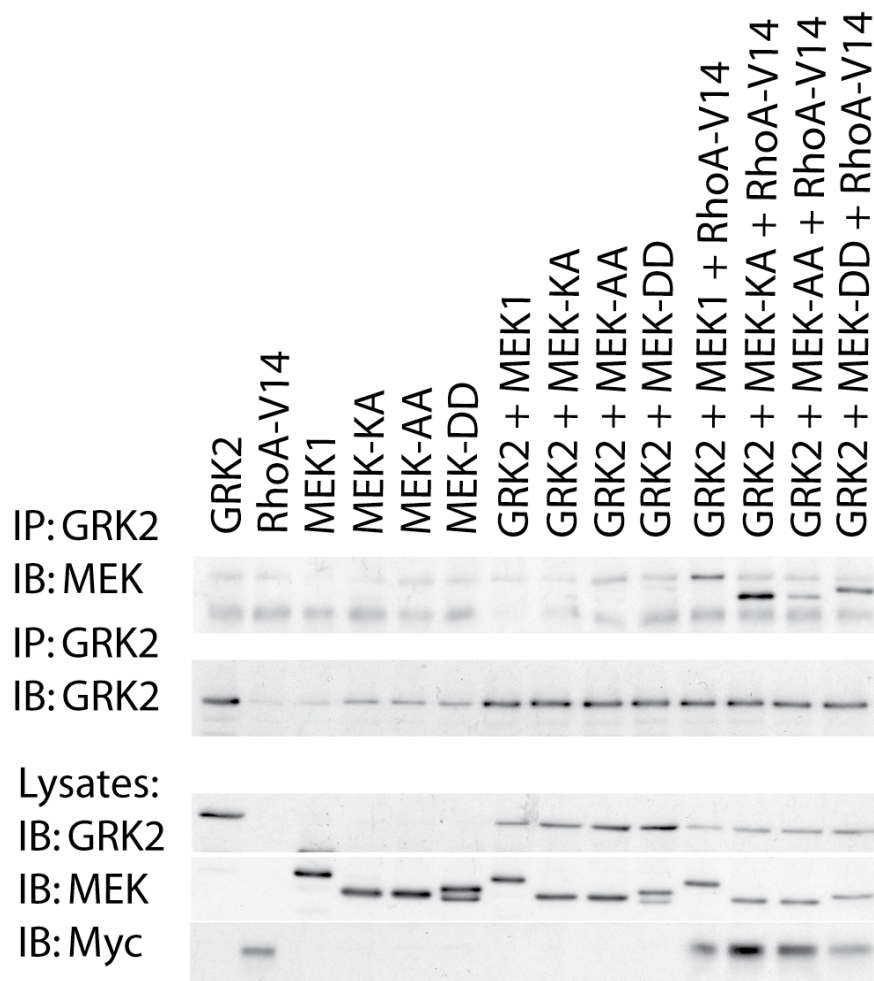


Figure 4.6. The Rho-dependent interaction of GRK2 with MEK1 is independent of the activation state of MEK1. Cos cells were transfected as indicated and protein expression confirmed by western blotting (lysates) with Myc-RhoA-V14 expression confirmed using an anti-Myc antibody (rabbit). GRK2 was immunoprecipitated (IP) and immunoprecipitates were blotted (IB) with an anti-MEK antibody. Western blots representative of 3 separate experiments.

4.6. RhoA-V14-dependent MEK1 and ERK2 binding is specific to GRK2.

In section 3.2, I showed that RhoA_{GTP} binding to GRK2 is mediated by its catalytic domain, which is highly conserved between GRK family members. It is therefore maybe not surprising to find that RhoA_{GTP} binding is conserved between

members of the GRK2 and GRK4 subfamilies (Figure 3.3). In contrast, I have found that MEK1 binding to GRK2 is mediated by a binding site that spans the catalytic and carboxyl-terminal regions of the kinase (Figure 4.4). The carboxyl-terminal region of the GRKs is structurally quite diverse and so I wondered whether the other GRKs would bind to MEK1 and ERK2 in response to RhoA-V14 binding. I compared the ability of GRK2 and GRK5 to co-immunoprecipitate with MEK1 and ERK2 upon co-expression of RhoA-V14. Interestingly, while we know that GRK5 binds to RhoA-V14, I found that, unlike GRK2, it fails to co-immunoprecipitate with MEK1 and ERK2 in response to RhoA-V14 expression (Figure 4.7). In Figure 4.7A, RhoA-V14 expression is shown to promote the formation of a complex between GRK2 and MEK1 but not between GRK5 and MEK1. Similarly, in Figure 4.7B, ERK2 can be seen to co-immunoprecipitate with GRK2 in response to RhoA-V14 expression but again expression of RhoA-V14 fails to promote GRK5 binding to ERK2. Thus, while RhoA-V14 binding is common to all of the GRKs, Raf/MEK/ERK scaffolding in response to RhoA-V14 binding may be a specific effector function of GRK2. This likely reflects the location of the MEK1 binding site within the poorly conserved carboxyl-terminal region of GRK2 and raises the question of what the effector functions of the other GRKs may be. Perhaps they act as scaffold proteins for other MAPK signalling pathways in response to Rho_{GTP} binding. This is the case for another group of MAPK scaffold proteins, the β -arrestins, where β -arrestin-1 and 2 can act as scaffolds for the ERK MAPK cascade while β -arrestin-2, but not β -arrestin-1, is also a scaffold for the JNK3 MAPK cascade (DeWire et al., 2007).

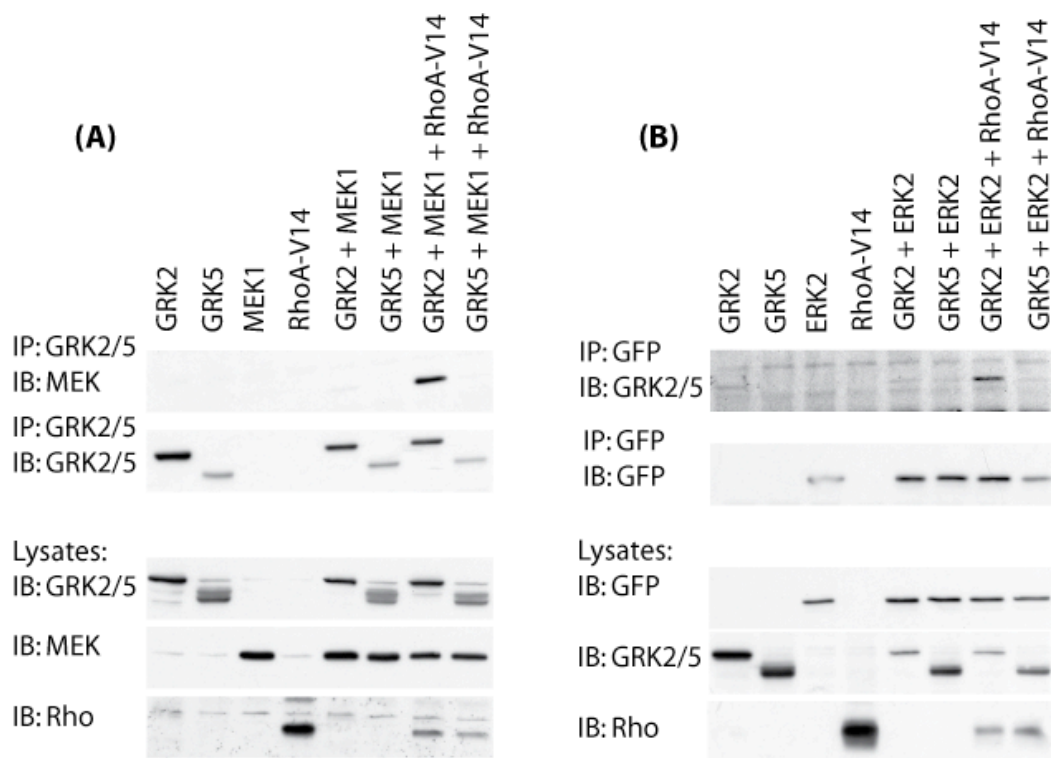


Figure 4.7. RhoA-V14-dependent MEK1 and ERK2 binding is specific to GRK2. Cos cells were transfected as indicated and protein expression confirmed by western blotting (lysates). **(A)** GRK2 or GRK5 were immunoprecipitated (IP) and immunoprecipitates were probed (IB) with an anti-MEK antibody or with an anti-GRK2-3 or GRK4-6 antibody to check IP efficiency. **(B)** GFP-ERK2 was immunoprecipitated (using a GFP antibody) and immunoprecipitates were probed (IB) using anti-GRK2 and GRK4-6 antibodies or with an anti-GFP antibody to check IP efficiency. Western blots are representative of 3 separate experiments.

4.7. Summary.

In summary, the data in this chapter demonstrates that RhoA_{GTP} binding to GRK2 promotes GRK2/Raf1, GRK2/MEK1 and GRK2/ERK2 complex formation. RhoA-N19 and RhoA-V14/F25N fail to interact with GRK2 or promote GRK2 binding to MEK1 or ERK2, supporting a novel role for GRK2 as a Rho-effector that acts as a scaffold protein for the ERK MAPK pathway in response to Rho_{GTP} binding. I have mapped the MEK1 binding site to a region on GRK2 that spans the catalytic and carboxyl-terminal domains. The carboxyl-terminal and catalytic domains of GRK2 in isolation can interact with MEK1 in the absence of RhoA-V14, however full length GRK2 requires RhoA-V14 binding to do so. Presumably, RhoA-V14 binding acts to expose a MEK1 binding site in GRK2 (and possibly binding sites for ERK2 and Raf1). In support of this, GRK2-W643A, which has a disrupted carboxyl-terminal region, can bind to MEK1 in the absence of RhoA-V14. This mutation may partly mimic the conformational change in GRK2 that would otherwise be mediated by Rho_{GTP} binding.

In chapter 3 I proposed the requirement for a conformational change in GRK2 in order to expose Rho_{GTP} binding sites within the catalytic domain of GRK2. This may be mediated by initial binding of Rho_{GTP} to another site within the amino-terminal region in GRK2 that would serve to promote the conformational change. Based on the results in this chapter, I propose an extension to the working model in which, exposure of the GRK2 catalytic domain for full Rho_{GTP} binding directs a further conformational change in GRK2 to expose a MEK1 binding site in the carboxyl-terminal/catalytic region. GRK2 may then act as a scaffold for the ERK MAPK pathway (Figure 4.8). The potential ERK scaffolding function of GRK2 is at

this stage only a hypothesis, as I have not demonstrated whether GRK2 can bind to all of the components of the ERK cascade simultaneously. I next wanted to investigate whether scaffolding of the ERK MAPK cascade by GRK2 can actually regulate ERK activation and whether this may be important in physiology.

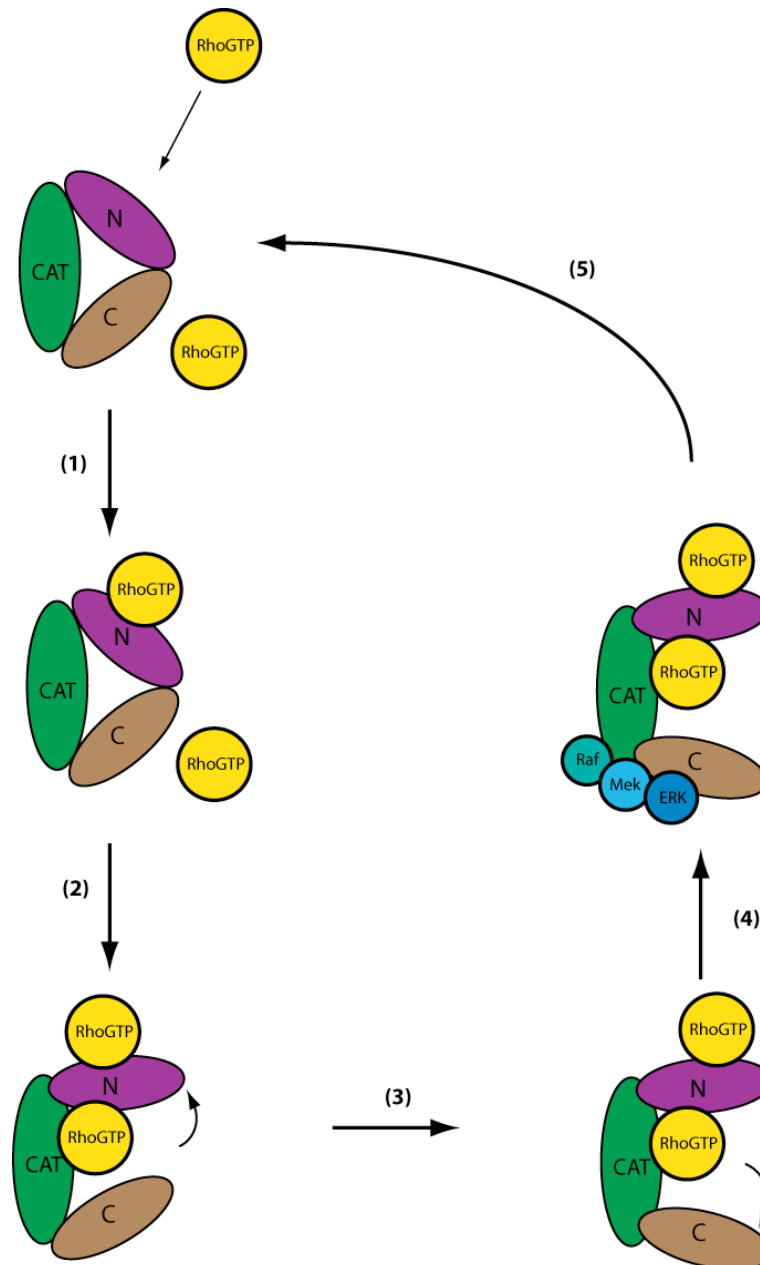


Figure 4.8. Working model of GRK2 scaffolding of the ERK MAPK cascade in response to Rho_{GTP} binding. 1) Schematic based on GRK2 crystal structures, which show the amino-terminal (N), catalytic (CAT) and carboxyl-terminal (C) domains form a triangle shape with extensive contacts between the amino-terminal RH domain and the carboxyl-terminal domain mediated by a hydrophobic patch and ionic interactions. 2) Rho_{GTP} binding to the amino-terminal domain promotes a conformational change enabling full Rho_{GTP} binding to the catalytic domain. 3) Rho_{GTP} binding in the catalytic domain results in another conformational change that exposes binding sites for MEK1 (and possibly ERK2 and Raf1) in the catalytic/carboxyl-terminal region (4). Rho_{GTP} hydrolysis to Rho_{GDP} and dissociation from GRK2 may result in the release of Raf, MEK and ERK (5).

5. GRK2 promotes ERK activation in response to EGF in HEK-293 cells and is required for EGF-induced proliferation of cultured Vascular Smooth Muscle Cells

5.1. Over-expression of GRK2 potentiates EGF-induced ERK activation in HEK-293 cells.

In chapter 4, I demonstrated that RhoA_{GTP} binding to GRK2 promotes GRK2 binding to Raf1, MEK1 and ERK2 and proposed that GRK2 may function as a Rho-activated scaffold protein for the ERK MAPK cascade. I next wanted to investigate whether Rho-dependent binding of Raf1, MEK1 and ERK2 by GRK2 can promote ERK activation in cells and, if so, whether this function of GRK2 has a role in physiology.

As discussed in detail in section 1.1.7, GRK2 is known to regulate ERK activation both positively and negatively via a number of different mechanisms. For example ERK activation in response to chemokines is negatively regulated by GRK2 interacting with and sequestering MEK (Jurado-Pueyo et al., 2008; Kleibeuker et al., 2008), while GRK2 binding to $\text{NF}\kappa\text{B1p105}$ in mouse peritoneal macrophages negatively regulates ERK activation in response LPS (Patil et al., 2011). In some circumstances, GRK2 can positively regulate ERK signalling. For example, over-expression of GRK2 in HEK-293 cells has been shown to potentiate EGF-mediated ERK activation (section 1.1.7), although the mechanism by which

this occurs is not fully understood. Rho is also activated downstream of the EGF receptor, for example via phosphorylation and activation of the Rho GEF Vav-2 (Pandey et al., 2000). I therefore decided to investigate whether positive regulation of ERK activation by GRK2 in response to EGF in HEK-293 cells may, at least in part, be mediated by a function of GRK2 as a Rho-activated ERK MAPK scaffold protein.

I measured ERK activation in response to EGF in HEK-293 cells by western blotting for phosphorylated ERK (P-ERK) as described in chapter 2 using an antibody that specifically recognises ERK1/2 phosphorylated at activatory threonine 202 and tyrosine 204 within the TEY motif of its activation loop. Treatment of HEK-293 cells with EGF results in a peak of ERK activation 2-5 min after EGF addition. 10 min after EGF stimulation, ERK activation is reduced back to basal levels (Figure 5.1A and B). When these cells are transfected with 1 ug of GRK2, ERK activation in response to EGF is potentiated by about 2-fold at each time point (Figure 5.1A and B). ERK activation is reduced to basal levels after 10 min, indicating that GRK2 can potentiate EGF-mediated ERK activation in HEK-293 cells without affecting the overall timecourse of ERK phosphorylation.

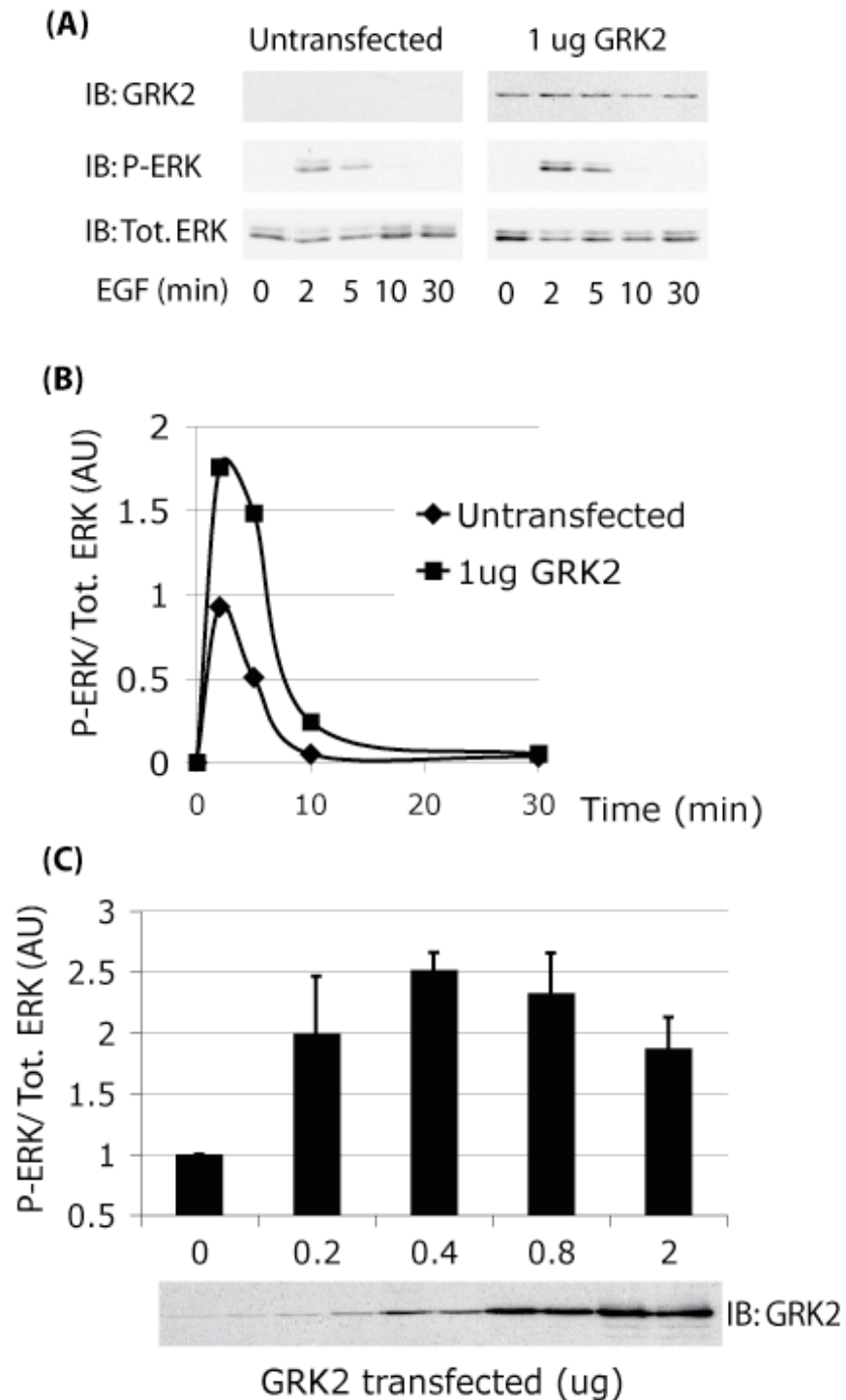


Figure 5.1. Over-expression of GRK2 potentiates EGF-induced ERK activation in HEK-293 cells. HEK-293 cells in 9 cm plates were transfected or not with the indicated amounts of GRK2. 24 h later, the cells were split onto 12-well plates and then serum starved overnight before treatment with EGF (200 ng/ml) for the times indicated (all samples treated for 5 min in (C)). Lysates were blotted for levels of phospho- and total ERK and ERK activation was calculated by quantifying bands

using QuantityOne and dividing values for phospho-ERK by values for total ERK in each sample, representative of 3 separate experiments. In (B), this is expressed as arbitrary units and the results shown are from a representative experiment. In (C), all samples were treated for 5 min with EGF and values for P-ERK/Tot. ERK for each amount of GRK2 are plotted relative to P-ERK/Tot. ERK for untransfected cells. A representative western blot is shown indicating levels of GRK2 expression. Error bars represent standard deviation from the mean of 3 separate experiments.

When I transfected HEK-293 cells with a range of different amounts of GRK2, I noticed that increasing levels of exogenous GRK2 incrementally increased EGF-induced ERK activation up to a point but, at higher levels of GRK2, ERK activation began to reduce (Figure 5.1C). Thus, there appears to be a trend towards a bell-shaped curve of ERK activation as a function of amount GRK2 transfected. For example, transfection of 0.4 ug GRK2 resulted in a 2.5 ± 0.2 fold increase in ERK activation relative to untransfected cells after 5 min EGF treatment. However, ERK activation was only 1.8 ± 0.3 fold greater than untransfected cells upon transfection of 2 ug of GRK2 (Figure 5.1C). A bell-shaped curve of ERK activation as a function of protein concentration is thought to be indicative of a scaffold protein and might indicate a scaffold function for GRK2 downstream of EGF. Too high a scaffold concentration is proposed to inhibit signalling by binding to the endogenous components of the kinase cascade separately and sequestering them from each other.

5.2. Treatment of HEK-293 cells and β -arrestin knockout MEFs with EGF promotes the formation of a complex between GRK2 and activated ERK.

If GRK2 is indeed an EGF-activated scaffold protein for the ERK MAPK cascade, you would expect that treatment of HEK-293 cells with EGF would result in an interaction between GRK2 and activated ERK. To test this, I treated HEK-293 cells with EGF for 5 min and immunoprecipitated endogenous GRK2 from cell lysates. Endogenous P-ERK co-immunoprecipitated with endogenous GRK2 in response to EGF treatment (Figure 5.2A). No P-ERK was co-immunoprecipitated with GRK2 in a control sample treated with EGF but lacking addition of the anti-GRK2 antibody during the IP. These results indicate that EGF stimulation promotes a specific interaction between GRK2 and activated ERK in HEK-293 cells, supporting a role for GRK2 as an EGF-activated ERK MAPK scaffold protein.

β -arrestins, which can be recruited to GPCRs and some RTKs in a GRK2-dependent manner, are known to act in some situations as ERK MAPK scaffold proteins. I therefore wanted to test whether or not the scaffold function of GRK2 downstream of EGF is dependent on β -arrestins. To do this, I performed the experiment described above in β -arrestin knockout MEFs, in which β -arrestins-1 and 2 are genetically ablated. Endogenous P-ERK co-immunoprecipitated with endogenous GRK2 in response to EGF in these cells, as it did in HEK-293 cells, indicating that ERK MAPK scaffolding by GRK2 is both independent of β -arrestins and not cell type specific (Figure 5.2B).

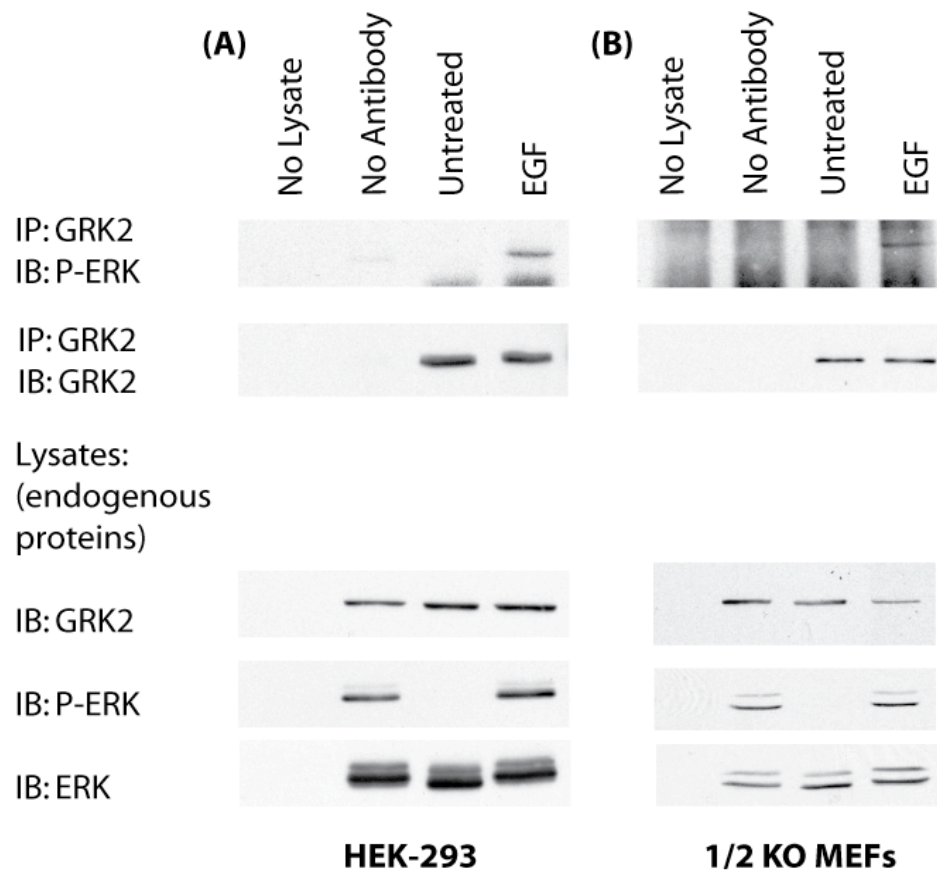


Figure 5.2. Treatment of HEK-293 cells and β -arrestin knockout MEFs with EGF promotes the formation of a complex between GRK2 and activated ERK. ~80% confluent HEK-293 cells and β -arrestin K/O MEFs were serum starved overnight and treated or not with EGF (200 ng/ml). Lysates were blotted for endogenous GRK2, ERK and P-ERK and GRK2 was immunoprecipitated (IP). Immunoprecipitates were probed (IB) with an anti-P-ERK antibody or an anti-GRK2-3 antibody to check IP efficiency. Western blots are representative of 3 separate experiments.

5.3. EGF-induced ERK MAPK scaffolding by GRK2 is Rho-dependent.

Figure 4.2 demonstrates that co-immunoprecipitation of Raf1, MEK1 and ERK2 with GRK2 is strongly potentiated by co-expression of RhoA-V14. I therefore hypothesised that, in line with our working model (Figure 4.8), ERK MAPK scaffolding by GRK2 in response to EGF in HEK-293 cells would be

dependent on active Rho. Inhibition of Rho should prevent GRK2 from binding to Raf, MEK and ERK and thus prevent GRK2 from potentiating ERK activation in response to EGF. Indeed, I found that pre-incubation of HEK-293 cells overnight with the Rho inhibitor C3 almost completely abolishes the effect of exogenous GRK2 on ERK activation (Figure 5.3A). Thus, ERK activation in cells treated with EGF for 5 min is increased by 2.3 ± 0.2 fold by transfection of 0.8 μ g GRK2 relative to untransfected cells. When cells are transfected with the same amount of GRK2 and treated with C3 however, EGF-induced ERK activation is not significantly different from untransfected cells. C3 treatment does not affect EGF-induced ERK activation in untransfected cells (data not shown). This data demonstrates that GRK2-dependent ERK activation downstream of the EGF receptor is Rho-dependent in HEK-293 cells.

Furthermore, co-expression of active RhoA-V14 and GRK2 in HEK-293 cells results in the association of more P-ERK with immunoprecipitated GRK2 following EGF treatment, as compared to GRK2 expression alone (Figure 5.3B). Thus, while inhibition of Rho reduces the effect of GRK2 on ERK activation, exogenous constitutively active RhoA promotes P-ERK binding by GRK2 in response to EGF in HEK-293 cells. Taken together, these results support a novel role for GRK2 as a Rho-dependent scaffold protein for the ERK MAPK cascade, downstream of the EGF receptor.

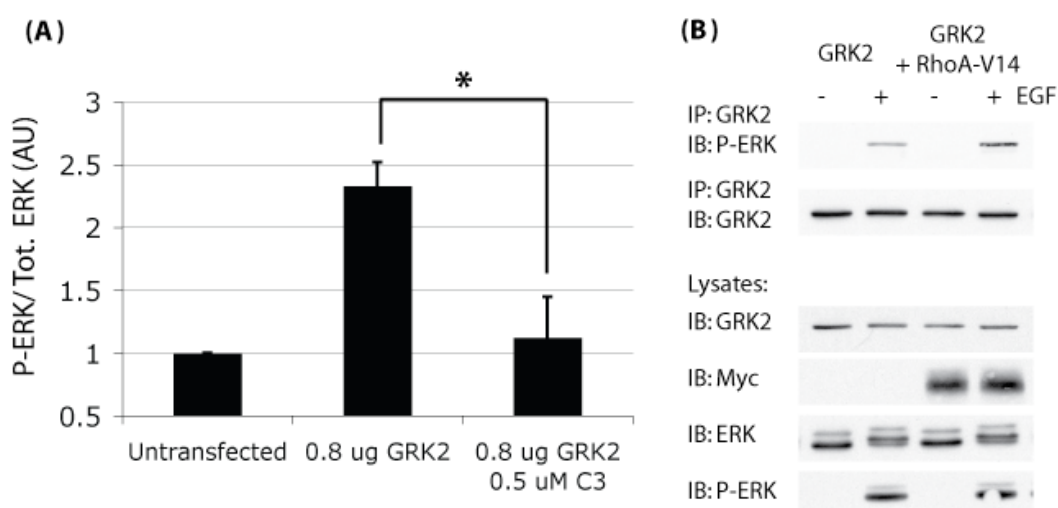


Figure 5.3. EGF-induced ERK MAPK scaffolding by GRK2 is Rho-dependent. **(A)** HEK-293 cells in 9 cm plates transfected or not with 0.8 μ g GRK2 were serum starved overnight in the presence or absence of 0.5 μ M C3 before treatment with EGF (200 ng/ml) for 5 min. Lysates were blotted for levels of phospho- and total ERK, bands were quantified using QuantityOne and ERK activation was calculated by dividing values for P-ERK by values for total ERK. The fold increase in EGF-induced ERK activation in response to GRK2 transfection is plotted relative to untransfected cells. Error bars represent standard deviation from mean of 3 separate experiments. * $P < 0.05$. **(B)** HEK-293 cells were transfected as indicated, serum starved overnight and treated or not with EGF (200 ng/ml) for 5 min. Protein expression was confirmed by western blotting. GRK2 was immunoprecipitated (IP) and immunoprecipitates probed (IB) for P-ERK, or GRK2 to check IP efficiency. Western blots are representative of 2 separate experiments.

5.4. EGF-induced ERK MAPK scaffolding by GRK2 is independent of GRK2 kinase activity.

I next wanted to test whether GRK2 kinase activity is required for its function as an ERK MAPK scaffold protein in response to EGF. In principle, kinase activity is not required for a protein to act as a scaffold as, by definition, a scaffold needs only to juxtapose the components of a signalling pathway in order to promote

their activation. It could also be the case however, that GRK2 phosphorylates one or more of the components of the Raf/ MEK/ ERK pathway and that this is required for its ability to promote ERK activation. GRK2 has been previously been shown to phosphorylate another MAPK, p38, resulting in inhibition of its activation (Peregrin et al., 2006) (section 1.1.7). I over-expressed Flg-tagged constructs of GRK2 or GRK2-K220R (a kinase dead mutant of GRK2) in HEK-293 cells and compared their ability to co-immunoprecipitate with activated ERK in response to EGF treatment. I found that P-ERK co-immunoprecipitated equally well with kinase dead GRK2 as with wildtype GRK2 in response to EGF, suggesting that GRK2 kinase activity is not required for its ability to act as an ERK MAPK scaffold protein downstream of the EGF receptor (Figure 5.4).

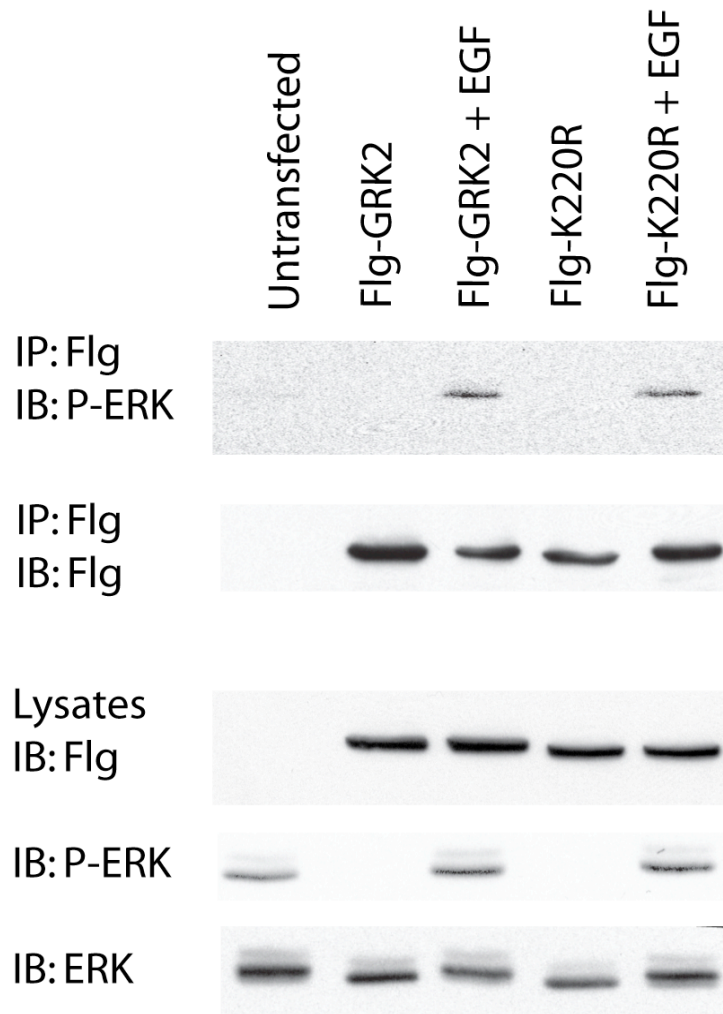


Figure 5.4. EGF-induced ERK MAPK scaffolding by GRK2 is independent of GRK2 kinase activity. HEK-293 cells were transfected as indicated, serum starved overnight and treated or not with EGF (200 ng/ml) for 5 min. Protein expression was confirmed by western blotting (lysates) and GRK2 was immunoprecipitated (IP) with a Flg antibody. Immunoprecipitates were probed (IB) for P-ERK, or with an anti-Flg antibody to check IP efficiency. Western blots are representative of 3 separate experiments.

5.5. Immunofluorescence experiments reveal the true extent of ERK activation mediated by GRK2 scaffolding.

In sections 5.1 to 5.4, I have presented evidence that suggests GRK2 potentiates ERK activation downstream of EGF by acting as a Rho-dependent scaffold protein. I next wanted to investigate how ERK scaffolding by GRK2 might affect the downstream functions of ERK in cells. ERK has over 100 known substrates that influence many different cellular processes. Scaffold proteins can in some cases direct specificity by determining the sub-cellular localisation of ERK activation (section 1.2). I therefore performed indirect immunofluorescence experiments in an attempt to assess where in cells ERK is activated in response to EGF and whether this is altered by the scaffold function of GRK2 when GRK2 is over-expressed.

Firstly, I transfected HEK-293 cells with 1 ug GRK2 and treated with a time-course of EGF. I then fixed and labelled the cells for GRK2 and P-ERK and imaged them using a confocal microscope as described in chapter 2. In agreement with the time-course of ERK activation observed by western blotting for P-ERK (Figure 5.1A), immunofluorescence images show a peak of EGF-induced ERK activation at 2-5 min, which is reduced to basal levels after 10 min (Figure 5.5). Western blotting experiments indicated that GRK2 transfection potentiates EGF-induced ERK activation by about 2-fold relative to untransfected cells (Figure 5.1A). Notably, immunofluorescence experiments show that cells in which GRK2 is over-expressed have a much greater than 2-fold increase in ERK activation relative to the surrounding untransfected cells (Figure 5.5). The effect of GRK2 on ERK activation in response to EGF is clearly underestimated by western blotting for P-ERK. This is

due to the fact that most of the cells that make up the total cell lysate do not over-express GRK2 (typical transfection efficiency was 20%) and will hence have lower levels of P-ERK following treatment with EGF. Only by immunofluorescence can the true effect of GRK2 on ERK activation be observed at a single cell level.

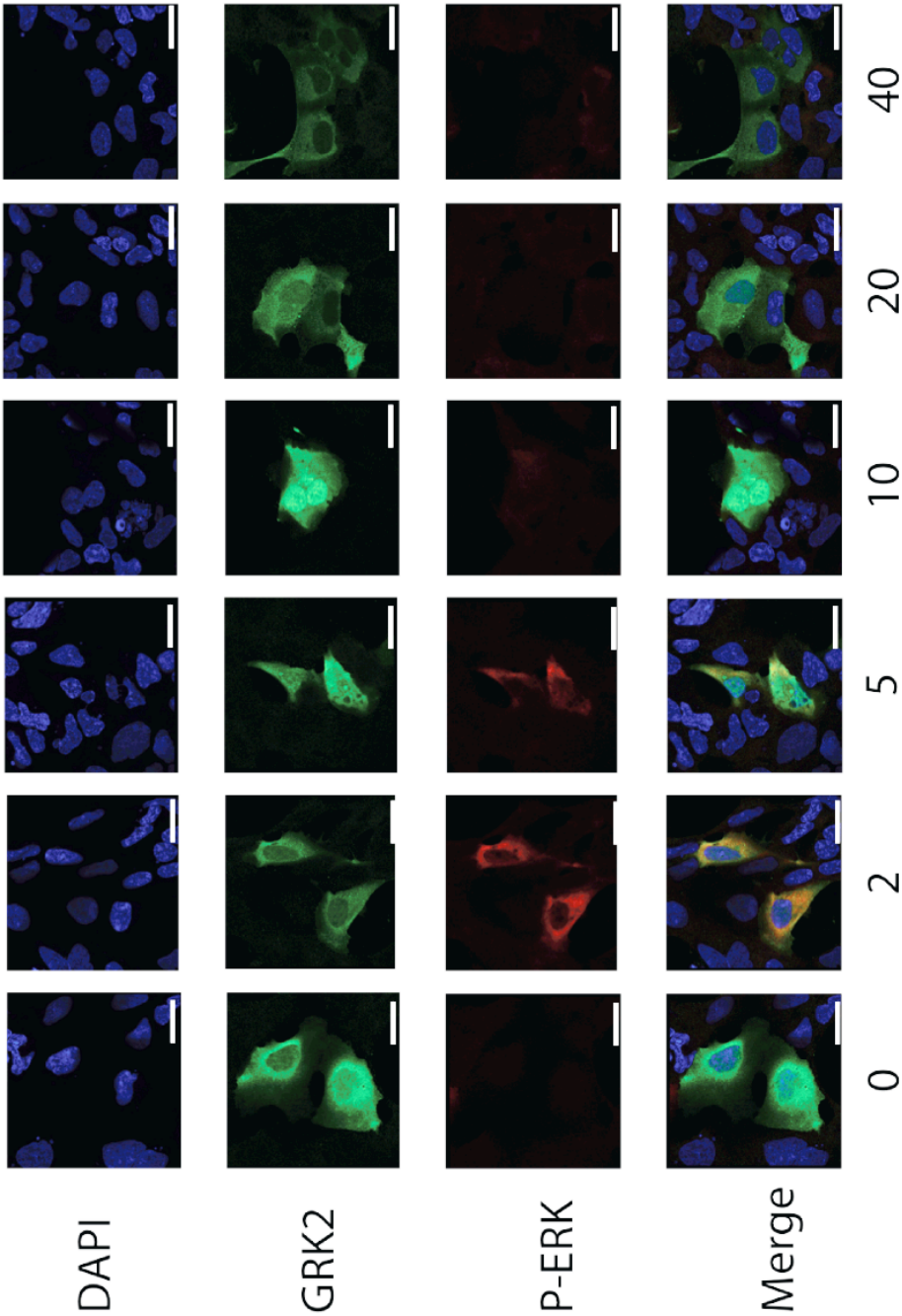


Figure 5.5. GRK2 translocates to the nucleus in HEK-293 cells treated with EGF. Confocal microscopy of HEK-293 cells transfected with GRK2 (1 ug), serum starved for 24 h and treated for the indicated times (in minutes) with EGF (200 ng/ml). Cells were labelled with an anti-GRK2 (green), an anti-P-ERK antibody (red) and Hoechst stain (blue). Scale bar 25 um.

Intriguingly, EGF treatment apparently promotes transient nuclear accumulation of GRK2. Following 10 min EGF treatment, significant amounts of GRK2 can be observed in the nucleus of HEK-293 cells (see Figure 5.5, 4th panel from the left in row labelled GRK2). This was surprising as, while nuclear localisation sequences have been found in GRKs 4, 5 and 6 (Johnson, PhD thesis 2007), none have been found in GRK2 and GRK2 has not previously been found to translocate to the nucleus. Furthermore, the time course of GRK2 nuclear translocation matches that previously reported for over-expressed GFP-ERK in response to EGF treatment in HEK-293 cells (Tilley et al., 2009). Thus, while active ERK is cytosolic 2-5 min after EGF treatment (see Figure 5.5, 2nd and 3rd panel from the left in row labelled P-ERK), translocation of GRK2 to the nucleus after 10 min may indicate that EGF-stimulated Raf/MEK/ERK scaffolding by GRK2 results in translocation of GRK2 in complex with Raf, MEK and ERK to the nucleus at this timepoint.

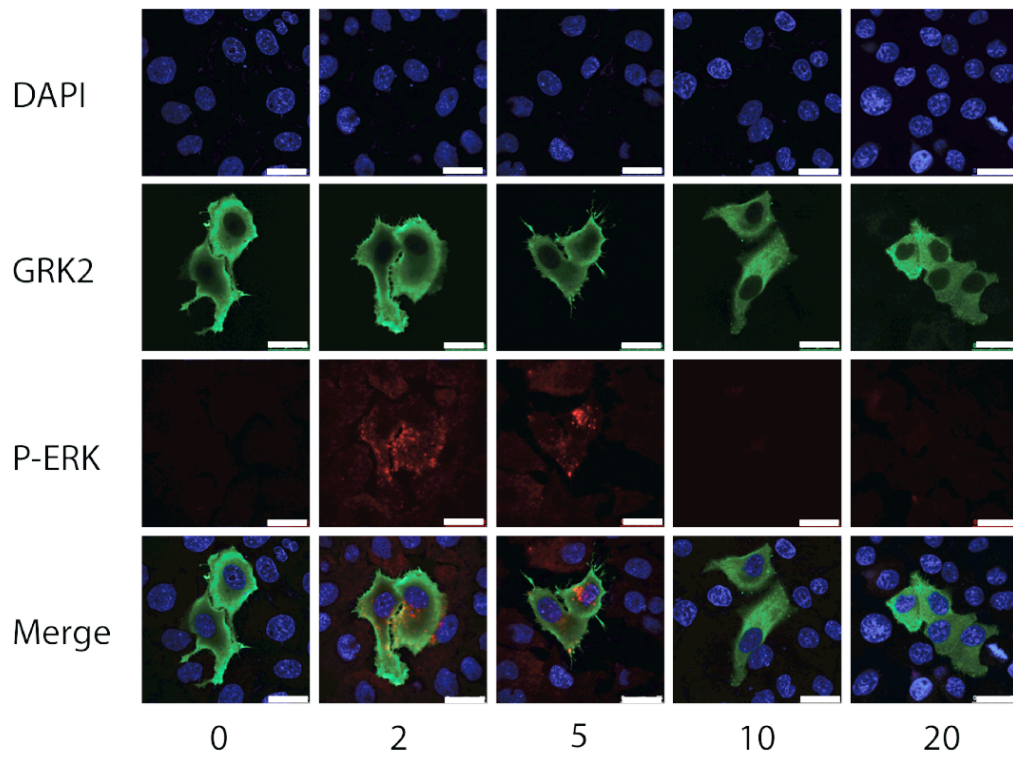
ERK is classically activated by phosphorylation at threonine and tyrosine residues within its TEY motif. The phospho-ERK antibody used in Figure 5.5 is raised against these sites and shows no signal 10 min after EGF addition (Figure 5.5, 4th panel from left in row labelled P-ERK). Thus, when GRK2 is in the nucleus, ERK is not phosphorylated at the classical activation sites and we don't know for sure whether ERK is nuclear. However, after 10 min, ERK may be phosphorylated at other sites that have been shown to promote nuclear accumulation of ERK

(Lorenz et al., 2009; Plotnikov et al., 2011), although we do not yet have antibodies against these sites. It would be interesting to investigate whether GRK2 mediates phosphorylation of ERK at these alternative phosphorylation sites. In addition, the formation of this complex and its subsequent translocation to the nucleus in response to EGF may be important to bring GRK2 into contact with potential nuclear substrates (discussed further in section 6.1).

The immunofluorescence experiments performed in HEK-293 cells reveal the true extent of GRK2 scaffolding on promoting ERK activation and also suggest that ERK scaffolding by GRK2 in response to EGF may be important for promoting nuclear accumulation of GRK2 and potentially ERK. I next performed further immunofluorescence experiments in Hep2 cells to assess whether EGF stimulation would result in similar distributions of P-ERK and GRK2 in a different cell type. Again, the images show that cells expressing exogenous GRK2 have a much greater than 2-fold increase in ERK activation relative to the surrounding untransfected cells (Figure 5.6A). This is also the case when the kinase dead GRK2-K220R mutant is over-expressed (Figure 5.6B), further supporting the evidence in figure 5.4, which indicated that GRK2 kinase activity is not required for its ability to act as an ERK MAPK scaffold protein downstream of the EGF receptor. In these cells however, there is no indication that ERK scaffolding by GRK2 in response to EGF promotes nuclear accumulation of GRK2. Rather, GRK2 appears slightly more accumulated at the plasma membrane after 2 min and 5 min EGF treatment before returning to a more uniform cytoplasmic distribution after 10 min (Figure 5.6A and 5.6B). No GRK2 can be seen in the nucleus at any time point. Also in contrast to the HEK-293 images, where activated (TEY-phosphorylated) ERK is distributed quite evenly throughout the cell at 2 min and 5 min stimulation, in Hep2 cells, the activated ERK

is more punctate at these time points with some puncta localized towards the plasma membrane and others more peri-nuclear (Figure 5.6A and 5.6B). Clearly GRK2 is capable of potentiating EGF-induced ERK activation in different cell types and to a greater extent than suggested by western blotting however, the distribution of GRK2 and P-ERK within the cell following EGF receptor activation is quite different in Hep2 to that seen in HEK-293 cells.

(A) GRK2 Wildtype



(A) GRK2-K220R

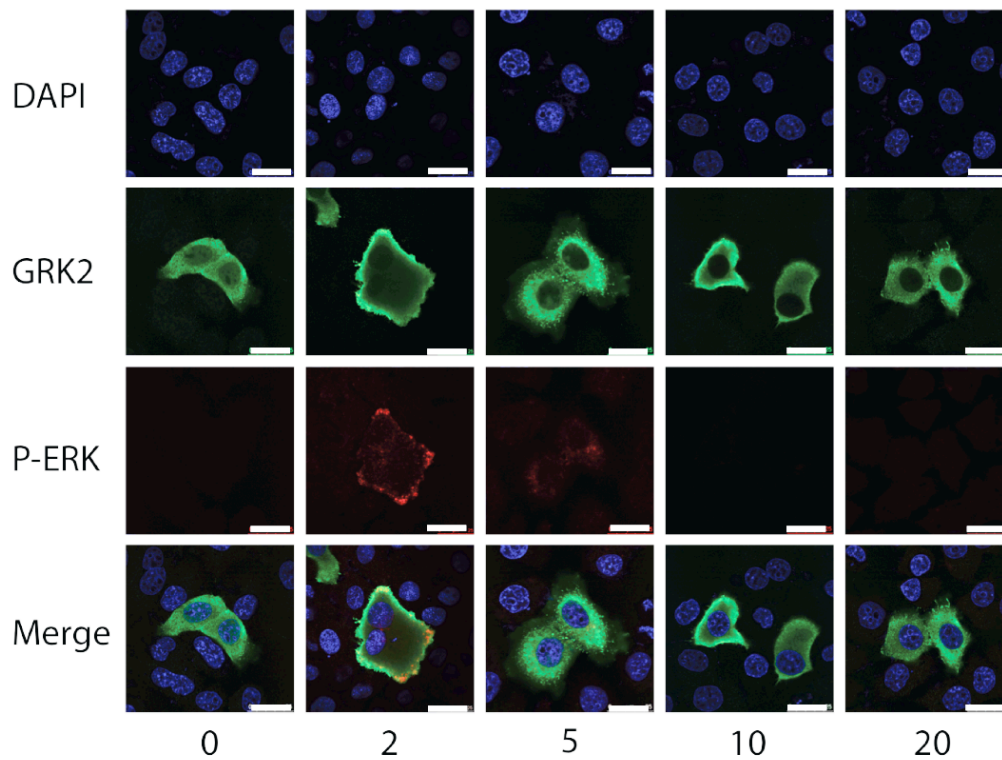


Figure 5.6. ERK scaffolding by GRK2 in response to EGF in Hep2 cells. Confocal microscopy of Hep2 cells transfected with GRK2 wildtype in (A) and GRK2-K220R in (B) (1 ug), serum starved for

24 h and treated for the indicated times (in minutes) with EGF (200 ng/ ml). Cells were labelled with an anti-GRK2 antibody (green), an anti-P-ERK antibody (red) and Hoescht stain (blue). Scale bar 25 um.

5.6. GRK2 is required for EGF-induced proliferation of VSMCs.

I next wanted to investigate whether the role of GRK2 as a Rho-activated ERK scaffold downstream of EGF is important in physiology. As discussed in section 1.3.2, several studies have linked increased levels of GRK2 with hypertension in both patients and animal models. Hypertension is associated in part with physiological changes in the vessel wall including excessive growth and proliferation of VSMCs. Eckhart and co-workers have developed a transgenic mouse model with GRK2 specifically over-expressed in the vascular smooth muscle (Eckhart et al., 2002). The mice have increased blood pressure associated with vascular thickening and eventually die of heart failure but the mechanism by which GRK2 causes these pathologies is not well understood (section 1.3.2). Abberant EGF receptor signalling (Yogi et al., 2010) and Rho activity and expression levels (section 1.4) (Loirand and Pacaud, 2010) have also been linked to hypertension. VSMCs cultured from salt-sensitive hypertensive rats have increased EGF receptor expression and display increased ERK activation in response to EGF treatment, relative to salt resistant controls (Ying and Sanders, 2005). I therefore decided to investigate whether Rho-activated ERK scaffolding downstream of the EGF receptor might in part explain how high levels of GRK2 cause hypertension.

To test this I cultured VSMCs from rat aortas and performed thymidine incorporation experiments as described in chapter 2 as a measure of their proliferation rate. Treatment with EGF for 24 h results in a ~2 fold increase in

thymidine incorporation relative to untreated cells (Figure 5.7A). 24 h treatment with serum typically results in a ~3 fold increase in thymidine incorporation (Figure 5.7A). Depletion of GRK2 levels in the cells by nucleofecting GRK2 siRNA typically resulted in a ~70% reduction in GRK2 levels (Figure 5.7B inset). Thymidine incorporation in VSMCs depleted of GRK2 was reduced by $59 \pm 12\%$ relative to cells nucleofected with a scrambled control (Figure 5.7B). This result indicates that GRK2 is required for EGF-induced proliferation of VSMCs. Furthermore, pre-treatment of the cells for 24 h with the Rho inhibitor C3 or with the MEK1 inhibitor PD184352 results in $49 \pm 13\%$ and $62 \pm 6.2\%$ respective reductions in thymidine incorporation, indicating that VSMC proliferation is also, at least in part, Rho and ERK dependent (Figure 5.7C). Taken together, this data supports a role for Rho-activated scaffolding of the ERK MAPK cascade by GRK2 downstream of EGF in mediating VSMC proliferation and this may in part explain how a high level of GRK2 in the vascular smooth muscle of mice results in vascular wall thickening and thereby high blood pressure. Clearly more work is needed to characterise the role of Rho-activated scaffolding of the ERK MAPK cascade by GRK2 in the vasculature, as discussed in section 6.6.

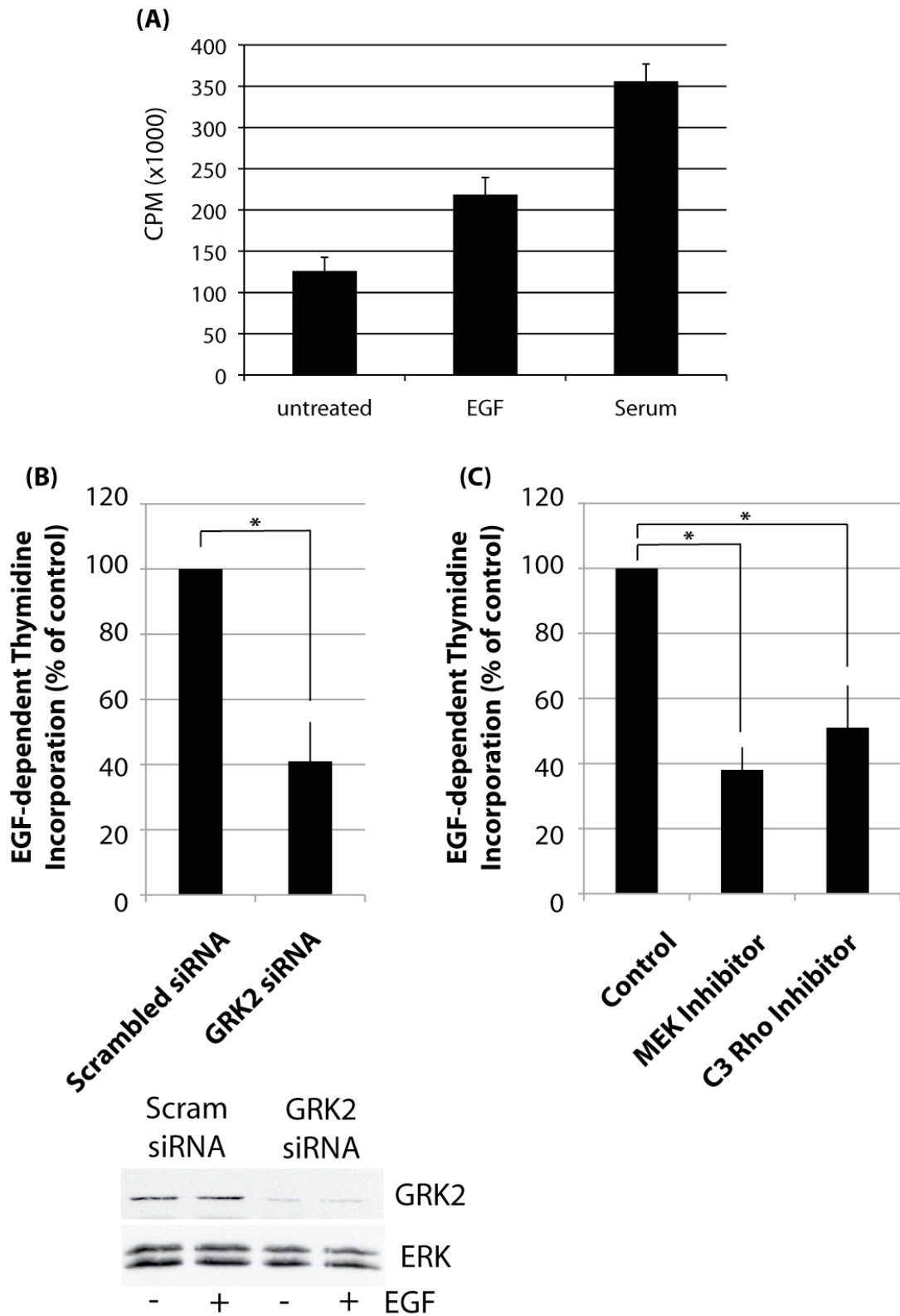


Figure 5.7. GRK2 is required for EGF-induced proliferation of VSMCs. (A) VSMCs were serum starved for 24 h and treated with EGF (10 ng/ml) or serum for 24 h in the presence of [3 H]-thymidine (3 uCi/ml). (B and C) VSMCs were nucleofected or not with GRK2 siRNA or scrambled control

(100 nM). After 24 h the cells were serum starved for 24 h and then treated with EGF (10 ng/ml) for a further 24 h in the presence of [³H]-thymidine (3 uCi/ml) with or without pre-treatment with the MEK inhibitor PD184352 (1 uM) or the Rho inhibitor C3 (0.5 uM). After 24 h EGF treatment, cells were harvested and counted for incorporation of [³H]-thymidine as described in chapter 2. Fold increase in [³H]-thymidine incorporation is plotted as a percentage of untreated control (for MEK inhibitor and C3 data) or scrambled control (for GRK2 siRNA data). Error bars represent standard deviation from the mean of 3 separate experiments. Statistical significance was assessed using the student's two-sample T-test with the null hypothesis proposing no difference between each condition and the control, * P<0.01.

5.7. Tyrosine phosphorylation of GRK2 downstream of EGF promotes its interaction with Rho and is required for its ability to act as an ERK scaffold.

Phosphorylation of GRK2 at tyrosine residues 13, 86 and 92 is known to promote the interaction of GRK2 with binding partners such as Gq (section 1.1.3) and GIT1 (section 1.1.7). The same tyrosine residues are also phosphorylated on GRK2 downstream of the EGF receptor (Chen et al., 2008). Knowing that GRK2 can function as an ERK scaffold in response to Rho-binding downstream of the EGF receptor, I wondered whether tyrosine phosphorylation of GRK2 in response to EGF receptor activation might promote GRK2 binding to Rho_{GTP} in order to enable ERK scaffolding to occur. This may explain how GRK2 positively regulates ERK activation downstream of the EGF receptor while negatively regulating it in response to other agonists such as ANG_{II} (Kim et al., 2005). Agonist-occupancy of the AT1R also activates Rho and so might be expected to promote ERK scaffolding by GRK2, however AT1R activation may not result in tyrosine phosphorylation of GRK2 (section 6.3).

I performed co-immunoprecipitation experiments in COS cells over-expressing GRK2, GRK2-K220R or GRK2 with Y13, 86 and 92 mutated to phenylalanine (GRK2-Y/F) with RhoA-V14. I found that 2.0 ± 0.3 fold and 2.5 ± 0.4 fold more GRK2 and GRK2-K220R respectively was co-immunoprecipitated with RhoA-V14 upon treatment with EGF, relative to untreated cells. These results indicate that EGF treatment promotes the interaction between GRK2 and Rho and that this interaction is independent of GRK2 kinase activity (Figure 5.8A, top panel and quantification in (B)). Importantly, while GRK2-Y/F can interact with RhoA-V14 (albeit more weakly than wildtype GRK2), its interaction with Rho is not potentiated by treatment with EGF (Figure 5.8A, top panel). Furthermore, the immunoprecipitates from cells over-expressing GRK2 or GRK2-K220R also contain activated ERK but activated ERK is not co-immunoprecipitated with Rho from cells in which GRK2-Y/F is over-expressed (Figure 5.8A, second panel from top). These results demonstrate that tyrosine phosphorylation of GRK2 at residues 13, 86 and 92 downstream of EGF is required to promote Rho binding and subsequent ERK scaffolding by GRK2. GRK2 kinase activity is not required but if GRK2 cannot be tyrosine phosphorylated, Rho binding is no longer promoted following EGF treatment and subsequent ERK scaffolding cannot occur. Thus, GRK2 may be able to act as a scaffold for ERK activation specifically in response to agonists that mediate tyrosine phosphorylation of GRK2 within its amino-terminal RH domain.

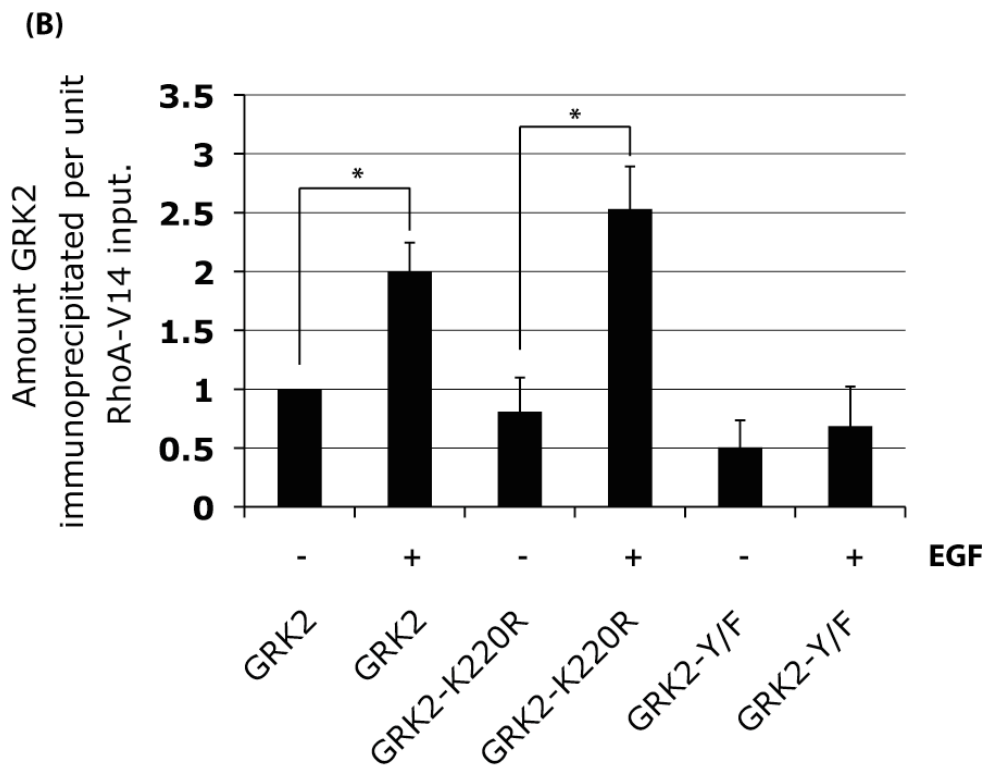
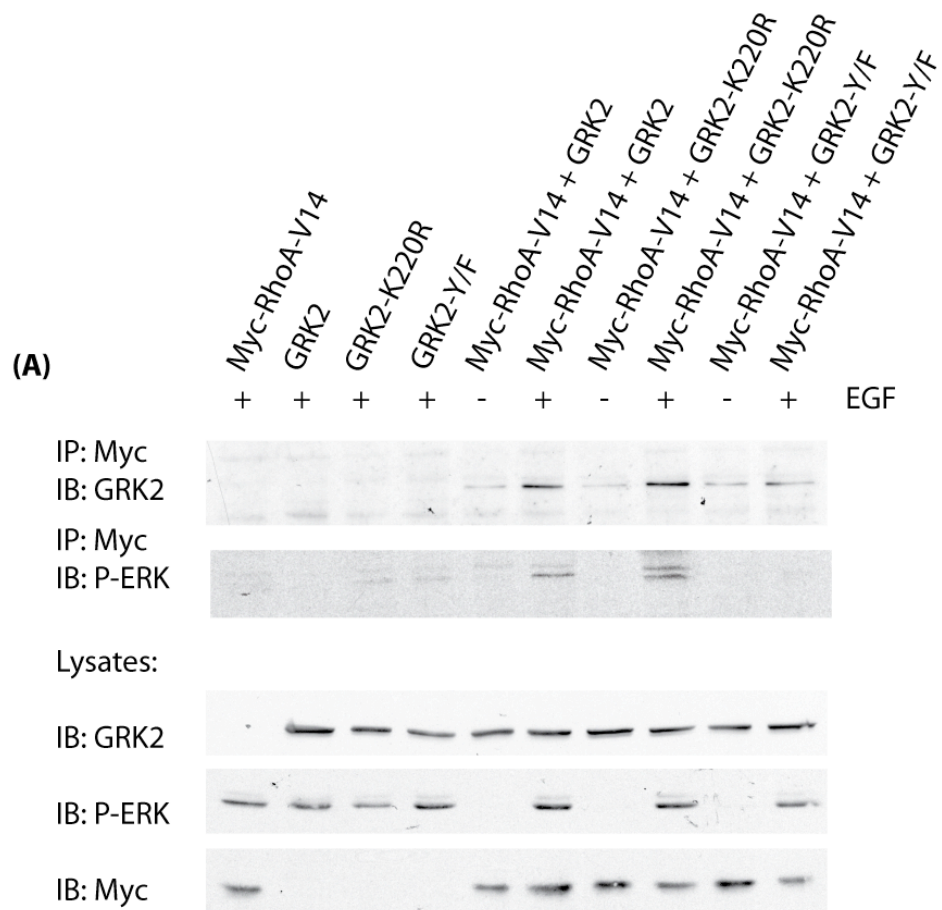


Figure 5.8. Tyrosine phosphorylation of GRK2 downstream of EGF promotes its interaction with Rho and is required for its ability to act as an ERK scaffold. (A) COS cells were transfected as indicated, serum starved for 24 h and treated or not with EGF (200 ng/ml). Protein expression was confirmed by western blotting (lysates) and Myc-tagged RhoA-V14 was immunoprecipitated (IP), using an anti-Myc antibody (rabbit). Immunoprecipitates were probed (IB) with anti-GRK2-3 and P-ERK antibodies. The western blot shown is a representative of 3 separate experiments. **(B)** Quantification of (A), amount of GRK2 co-immunoprecipitated with Myc-RhoA-V14 per unit Myc-RhoA-V14 expressed in lysates is plotted relative to amount of co-immunoprecipitation seen for wildtype GRK2, error bars represent standard error of the mean from 3 separate experiments. Statistical significance was assessed using the student's two-sample T-test with the null hypothesis proposing no difference between the treated and untreated condition, * $P < 0.05$.

5.8. Summary.

In summary, the data in this chapter demonstrates that GRK2 can promote ERK activation downstream of the EGF receptor by acting as a Rho-dependent scaffold protein. GRK2 kinase activity is not required. Tyrosine phosphorylation of GRK2, most likely at residues 13, 86 and 92, is required to promote GRK2 binding to Rho_{GTP} in response to EGF. In an extension to the working model in Figure 4.8, I propose that tyrosine phosphorylation of GRK2 in the amino-terminal domain promotes Rho_{GTP} binding to this region (Figure 5.9 (1)). This directs a conformational change in GRK2 that exposes the catalytic domain for full Rho_{GTP} binding (Figure 5.9 (2)). This in turn induces a further conformational change that exposes binding sites within the catalytic/carboxyl-terminal region (Figure 5.9 (3)) required for subsequent scaffolding of the ERK MAPK cascade (Figure 5.9 (4)). This scaffolding role of GRK2 may have an important function in the vasculature. GRK2 is required for EGF-induced thymidine incorporation in VSMCs, which I hypothesise may in part explain why high levels of GRK2 are associated with vascular thickening and hypertension in patients and animal models (Cohn et al., 2009; Eckhart et al., 2002).

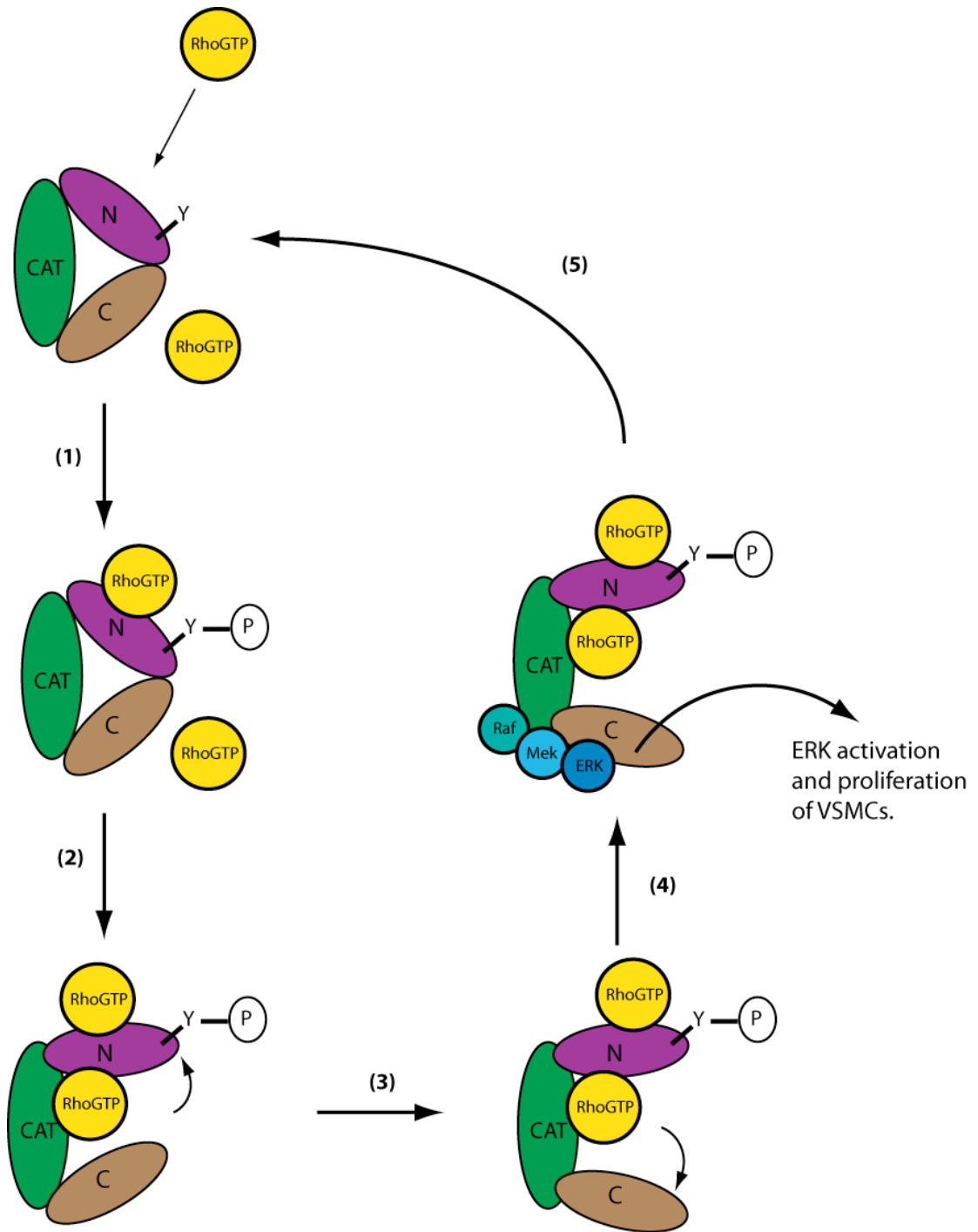


Figure 5.9. Extended working model for GRK2 scaffolding of ERK. 1) Schematic based on GRK2 crystal structures, which show the amino-terminal (N), catalytic (CAT) and carboxyl-terminal (C) domains form a triangle shape with extensive contacts between the amino-terminal RH domain and the carboxyl-terminal domain mediated by a hydrophobic patch and ionic interactions. RhoGTP binding to the GRK2 amino-terminal domain is promoted by tyrosine phosphorylation of GRK2 at

residues 13, 86 and 92. 2) Rho_{GTP} binding to the amino-terminal domain promotes a conformational change enabling full Rho_{GTP} to the catalytic domain. This may or may not involve another molecule of Rho_{GTP}. 3) Rho_{GTP} binding in the catalytic domain results in another conformational change that exposes binding sites for MEK1 (and possibly ERK2 and Raf1) in the catalytic/carboxyl-terminal region (4). This facilitates activation of ERK and is required for EGF-mediated proliferation of cultured VSMCs. 5) Dephosphorylation of amino-terminal tyrosine residues may cause Rho_{GTP} dissociation.

6. Discussion

The work presented in this thesis identifies Rho_{GTP} as a novel direct binding partner for GRK2. Rho_{GTP} binds to GRK2 within the catalytic domain of the kinase but this does not appear to affect its kinase activity or the ability of GRK2 to desensitise GPCRs. Rather, Rho_{GTP} binding to GRK2 promotes GRK2 binding to Raf1, MEK1 and ERK2, the three components of the ERK MAPK cascade. When over-expressed in HEK-293 cells, GRK2 potentiates ERK activation in response to EGF by acting as a Rho-dependent scaffold protein for the ERK MAPK cascade. Phosphorylation of GRK2 at tyrosine residues within its amino-terminal domain is required for the EGF-stimulated interaction of Rho_{GTP} and GRK2 and for subsequent P-ERK binding by GRK2 downstream of the EGF receptor. In VSMCs, GRK2 is required for EGF-induced, Rho- and ERK-dependent cell proliferation. Thus, Rho-activated ERK MAPK scaffolding by GRK2 may have important roles in the vasculature, where increased levels of GRK2 (section 1.3.2) and Rho (section 1.4) have separately been linked with hypertension. While Rho_{GTP} binds to all of the ubiquitously expressed GRKs, MEK1 and ERK2 binding in response to Rho_{GTP} may be specific to GRK2 as GRK5 fails to interact with MEK1 or ERK2 in the presence of RhoA-V14. Based on my results, I propose a speculative model in which activation of Rho and phosphorylation of GRK2 downstream of the EGF receptor promotes an interaction between GRK2 and Rho_{GTP}, allowing subsequent scaffolding of the ERK MAPK cascade by GRK2 (Figure 6.1).

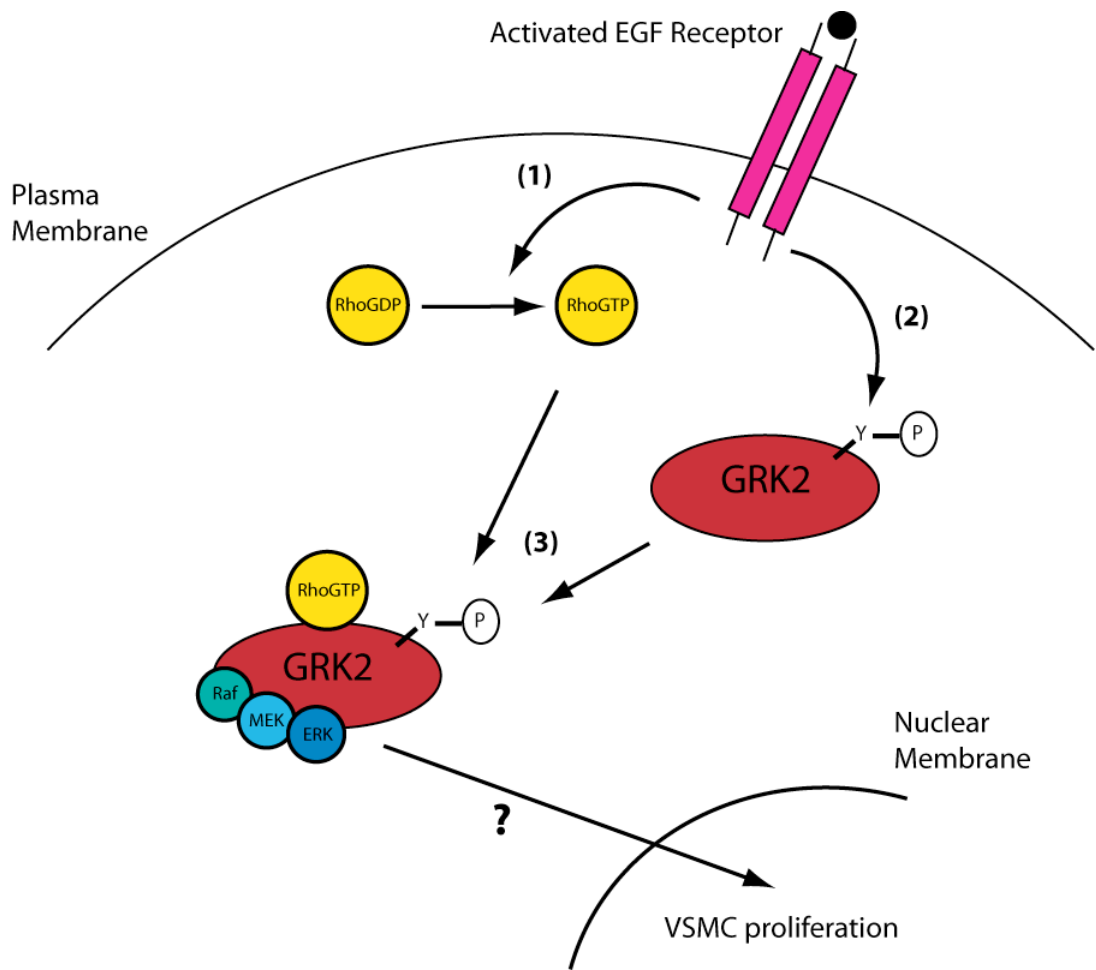


Figure 6.1. GRK2 is a Rho-dependent scaffold protein downstream of the EGF receptor. In response to agonist binding at the EGF receptor, Rho is activated (1) and GRK2 is tyrosine phosphorylated at residues within its amino-terminal region (2). This allows GRK2 to interact with Rho_{GTP} and subsequently scaffold the components of the ERK MAPK cascade (3). Preliminary data (discussed in text) suggests that ERK scaffolding by GRK2 may promote translocation of GRK2 and/or ERK into the nucleus. This pathway may be required for EGF-induced proliferation of VSMCs.

6.1. The ERK scaffolding function of GRK2.

Scaffolds have been described as ‘proteins that organise signalling complexes by binding at least two signalling components together and promoting their communication by proximity’ (Buday and Tompa). As such, scaffolding is important to ensure the integrity and specificity of signalling pathways. Scaffold proteins can function by determining the strength, duration or localisation of a signal. This is presumably particularly important for ERK1/2 signalling due to the large number of potential downstream effectors, often with opposing cellular functions, including cell proliferation and cell death (section 1.2).

β -arrestins are recruited to GPCRs in response to GPCR phosphorylation by GRKs, where they prevent further G protein activation by GPCRs and initiate receptor internalisation (section 1.1.2). β -arrestins thus function in concert with GRKs to desensitise GPCRs, however, they also act as multi-functional scaffold proteins for the activation of various signalling pathways, including ERK activation (DeWire et al., 2007). Here I show that GRK2, like the β -arrestins, can also act as an ERK MAPK scaffold protein. GRK2-mediated ERK scaffolding in response to EGF is independent of β -arrestins as EGF can promote the interaction of GRK2 with activated ERK in β -arrestin-knockout MEFs (Figure 5.2). Furthermore, GRK2-mediated and β -arrestin-mediated scaffolding of the ERK MAPK cascade differ in several respects, as detailed below.

β-arrestin-mediated ERK MAPK scaffolding

β-arrestin-2 co-immunoprecipitates with Raf1, MEK1 and ERK2 when all four proteins are co-expressed in COS cells (Luttrell et al., 2001). The formation of the complex between β-arrestin-2 and the three components of the ERK cascade is promoted by treatment of cells with GPCR agonists, such as ANG_{II} (Luttrell et al., 2001). β-arrestin-1 also acts as an ERK scaffold protein in response to agonist stimulation, at least downstream of the type 1 parathyroid hormone receptor (PTH1R) (Gesty-Palmer et al., 2006). In contrast to the transient ERK activation mediated by G proteins, β-arrestin-mediated ERK activation downstream of the AT1R peaks after 10 min stimulation and is sustained for up to 90 min (Ahn et al., 2004). β-arrestins are also recruited, in an agonist-dependent fashion, to some RTKs, including the IGF-1 receptor and the EGF receptor (Hupfeld and Olefsky, 2007). There is, however, no evidence that they act as ERK scaffolds downstream of RTKs. Another MAPK, JNK3, and its upstream activators: ASK1 and MKK4 have been found to co-immunoprecipitate with β-arrestin-2 (McDonald et al., 2000). Thus, β-arrestin-2 can also act as a scaffold protein for JNK3 activation. β-arrestin-2-mediated JNK3 activation, like β-arrestin-2-mediated ERK activation, promotes sustained activation of JNK3 downstream of the AT1R (McDonald et al., 2000).

Although the precise downstream substrates of β-arrestin-2-activated MAPKs have not been characterised, ERK scaffolding by β-arrestins is known to result in cytosolic retention of activated ERK on 'β-arrestin signalosomes' at the expense of ERK translocation to the nucleus (DeWire et al., 2007; Tohgo et al., 2003). While the wildtype AT1R stimulates ERK activation in the cytosol and in the nucleus, the DRY-AAY mutant of the receptor, which fails to couple to Gq and thus

can only activate ERK via β -arrestins, only activates cytosolic ERK with no detectable affect on transcription (Lee et al., 2008). In summary, β -arrestin-mediated ERK scaffolding downstream of GPCRs results in sustained activation of ERK and retention of ERK in the cytosol.

GRK2-mediated ERK MAPK scaffolding

GRK2 binding to Raf1, MEK1 and ERK2 is dependent on Rho_{GTP} , as co-immunoprecipitation of GRK2 with Raf1, MEK1 or ERK2 is strongly potentiated by co-expression of RhoA-V14 (Figure 4.2 D, E and F). In contrast, as described above, co-expression of β -arrestin-2 with Raf1, MEK1 or ERK2 in COS cells in the absence of any other exogenous proteins or stimuli is sufficient to enable co-immunoprecipitation of β -arrestin-2 with the three components of the cascade (Luttrell et al., 2001). Thus, *in vivo*, agonist stimulation and subsequent membrane recruitment of β -arrestins may be sufficient to enable β -arrestin-mediated ERK activation, however ERK scaffolding by GRK2 may require an additional stimulus in the form of activated Rho. Unlike β -arrestin-mediated ERK scaffolding, Rho-dependent ERK scaffolding by GRK2 downstream of the EGF receptor potentiates ERK activation while maintaining a rapid and transient timecourse of ERK activation (Figure 5.1A and B). I have not yet investigated whether or not ERK scaffolding by GRK2 occurs downstream of other RTKs or GPCRs (the specificity of GRK2 signalling is discussed in section 6.3). ERK scaffolding by β -arrestins has only been demonstrated to occur downstream of GPCRs, suggesting that ERK scaffolding by GRK2 and by β -arrestins may occur selectively downstream of different receptors.

In VSMCs, GRK2 is required for EGF-induced thymidine incorporation (Figure 5.7), suggesting that, in contrast to β -arrestins, ERK scaffolding by GRK2 may be important for nuclear rather than cytosolic functions of ERK. In support of this, immunofluorescence experiments performed in HEK-293 cells showed that, after 10 min EGF treatment, GRK2 translocates to the nucleus (Figure 5.5). Phosphorylation of ERK at its classically activating threonine and tyrosine residues within the TEY motif of the activation loop peaks at 2-5 min and these sites are predominantly dephosphorylated after 10 min. However, other groups have found that total ERK, like GRK2, translocates to the nucleus after 10 min EGF treatment in HEK-293 cells (Tilley et al., 2009). Thus, the timecourse of ERK nuclear translocation in response to EGF appears to match that of GRK2, at least in HEK-293 cells. Nuclear translocation of GRK2 was not, however, observed in response to EGF treatment in Hep2 cells (Figure 5.6). Therefore, EGF-induced nuclear translocation of GRK2 and ERK may be cell type specific. The subcellular localisation of ERK, P-ERK and GRK2 in VSMCs following EGF treatment requires further investigation (section 6.6)

Nuclear translocation of ERK

Nuclear translocation of ERK is thought to rely on phosphorylation of ERK at alternative sites to those within the TEY motif (Lorenz et al., 2009; Plotnikov et al., 2011). For example, in COS cells transfected with the M1 muscarinic receptor, 10 min treatment with carbachol results in nuclear translocation of ERK2. This is prevented when threonine 188, an ERK autophosphorylation site outside of the TEY motif, is mutated to alanine (ERK2-T188A) (Lorenz et al.,

2009). While ERK phosphorylation in the TEY motif is transient in response to carbachol (peaks at 2-5 min), phosphorylation at T188 increases for up to an hour after stimulation (Lorenz et al., 2009). In mouse hearts, trans-aortic constriction (TAC) promotes phosphorylation of cytosolic and nuclear substrates of ERK but in transgenic mice expressing the T188A mutant of ERK2, TAC fails to promote phosphorylation of nuclear substrates of ERK, while cytosolic substrates are unaffected (Lorenz et al., 2009). Thus, sustained T188 phosphorylation promotes nuclear localisation of ERK associated with phosphorylation of its nuclear substrates.

T188 autophosphorylation requires ERK1/2 dimerisation, mediated by its binding to G β γ . This occurs specifically downstream of Gq-coupled but not Gi-coupled receptors (Lorenz et al., 2009) but it is not clear exactly how this specificity is achieved. The ability of GRK2 to bind to Gq and G β γ (Tesmer et al., 2005) and also to act as a scaffold for ERK activation may position GRK2 for recruiting G β γ to activated ERK downstream of Gq-coupled receptors in order to promote ERK nuclear translocation. G β γ binding and T188 autophosphorylation of ERK can also occur in response to neuregulin-1- β 1 binding to the EGFR family member ErbB (Lorenz et al., 2009) and treatment of cardiomyocytes with neuregulin-1- β 1 was shown to result in phosphorylation and activation of Gq (Lorenz et al., 2009). Gq and G β γ -mediated nuclear localisation of ERK may operate downstream of other RTKs, including the EGF receptor, however, while the EGF receptor is known to couple to G β γ , Gs and G_{12/13} (Pyne and Pyne, 2011), there is currently no evidence that it couples to Gq, although this warrants further investigation.

Another group have identified Casein kinase 2 (CK2) as a kinase that phosphorylates ERK at serine residues 244 and 246, which lie in a nuclear

translocation sequence (NTS) (Plotnikov et al., 2011). Phosphorylation of these sites by CK2 promotes nuclear translocation of ERK and phosphorylation of its nuclear substrates (Plotnikov et al., 2011). GRK2 has a similar preference to CK2 for serine and threonine residues within acidic environments and CK2 also shares some substrates with GRK2, including GPCRs (Torrecilla et al., 2007). Thus, it is possible that GRK2 may also phosphorylate ERK at these sites to promote its nuclear localisation. When antibodies recognising ERK phospho-T188 and phospho-NTS become commercially available, it would be interesting to investigate whether GRK2 scaffolding of ERK affects phosphorylation at these sites. Kinase dead GRK2 co-immunoprecipitates with TEY-phosphorylated ERK in response to EGF treatment suggesting that GRK2 kinase activity may not be required for promoting ERK activation, however, GRK2 kinase activity may be required for nuclear localisation of ERK. It is possible that ERK scaffolding by GRK2, but not by other scaffolds such as β -arrestins, promotes phosphorylation of ERK at T188 and/or within the NTS to bias signalling towards nuclear rather than cytosolic substrates. If it is indeed the case that activated ERK translocates to the nucleus in complex with GRK2 in response to EGF, this may alternatively function to bring GRK2 into contact with potential nuclear substrates. Nuclear localisation sequences have been found in GRKs 4, 5 and 6 (Johnson, PhD thesis 2007) and GRK5 is known to have substrates within the nucleus, such as class II histone deacetylases (HDACs) (Martini et al., 2008).

Rho-activated scaffolding

As discussed above, one important difference between GRK2-mediated ERK scaffolding and scaffolding by other proteins such as the β -arrestins is that GRK2 is a regulated scaffold protein that specifically activates ERK only in the presence of activated Rho. Interestingly, another MAPK scaffold protein, connector enhancer of KSR-1 (CNK1) also appears to function in a Rho-dependent manner. CNK1 is a scaffold protein that can regulate a number of different signalling pathways including PI3K/Akt activation, Src-mediated Raf1 activation and JNK MAPK signalling (Fritz et al.; Ziogas et al., 2005). CNK1 has been identified as a target of Rho (Jaffe et al., 2004) and it seems that binding of active Rho by CNK1 specifically mediates its scaffolding of the JNK MAPK cascade (Jaffe et al., 2005). CNK1 and GRK2 both have many different roles in regulating signalling and how such multifunctional proteins can achieve signalling specificity in different situations is an interesting and poorly understood issue. In this case, it appears that CNK1 and GRK2 both use binding to active Rho to specifically promote MAPK activation. Signalling specificity downstream of GRK2 will be further discussed in section 6.3.

6.2. GRK2-mediated regulation of EGFR and PDGFR signalling.

GRK2 is recruited to agonist occupied EGF and PDGF receptors where it phosphorylates their intracellular domains (Freedman et al., 2002). This is analogous to the classical function of GRKs in GPCR desensitisation, however, while GRK2 recruitment to the PDGF receptor results in its desensitisation, this is not the case for

the EGF receptor (Hildreth et al., 2004) (sections 1.1.4 and 1.1.7). GRK2 recruitment to, and phosphorylation of, the PDGF receptor diminishes receptor activation, as measured by tyrosine phosphorylation, and reduces PDGF-induced activation of PI3K/Akt (Hildreth et al., 2004). GRK2 is phosphorylated at tyrosine residues including Y13, Y86 and Y92 downstream of the PDGF receptor, resulting in activation of GRK2 and therefore forming a negative feedback loop for PDGF signalling (Wu et al., 2005) (section 1.1.4).

GRK2 is also tyrosine phosphorylated at these sites in response to agonist binding to the EGF receptor. Although the EGF receptor is a substrate for GRK2 (Freedman et al., 2002), the activation state of the EGF receptor and EGF receptor-mediated PI3K/Akt activation are unaffected by GRK2 (Hildreth et al., 2004). Increased GRK2-mediated phosphorylation of opioid receptors in response to EGF (Chen et al., 2008) suggests that GRK2 is indeed activated downstream of the EGF receptor, as it is downstream of the PDGF receptor. Furthermore, cells expressing GRK2 Y13, 86, 92F (GRK2-Y/F), which has been reported (Penela et al., 2001) to have equivalent catalytic activity and sub-cellular distribution as wildtype GRK2, display reduced EGF-induced GRK2-dependent opioid receptor phosphorylation. This data therefore suggests that GRK2 is activated by tyrosine phosphorylation at residues Y13, Y86 and Y92 downstream of both the EGF and PDGF receptors, which in turn are both substrates for GRK2. However, reduced receptor activation and signalling to PI3K/Akt is a consequence only of GRK2-mediated PDGF receptor phosphorylation and not EGF receptor phosphorylation.

While PDGF receptor activation and signalling to PI3K is reduced by GRK2 phosphorylation of the receptor, PDGF-induced activation of ERK1/2 is unaffected (Hildreth et al., 2004). This suggests that PDGF receptor phosphorylation by GRK2

may prevent the interaction of the PDGF receptor with some of its downstream adaptor proteins and not others. The work of Lan Ma and co-workers (Gao et al., 2005b) as well as the work presented herein demonstrates that GRK2 can positively regulate ERK activation downstream of the EGF receptor. The fact that GRK2 can potentiate EGF induced ERK activation without affecting the activation state of the receptor *per se* supports a GRK2-dependent affect on ERK activation as opposed to a mechanism that simply potentiates EGF receptor signalling. I observe by western blotting (Figure 5.1A) and by immunofluorescence (Figure 5.5) that over-expression of GRK2 in HEK-293 cells potentiates ERK activation in response to EGF. I find GRK2 in complex with activated ERK in cells treated with EGF (Figure 5.2) and this interaction is Rho-dependent (Figure 5.3). Thus, the data presented in this thesis suggests that the ability of GRK2 to potentiate ERK activation downstream of the EGF receptor is due to its function as a Rho-activated ERK MAPK scaffold protein.

A different group have found that EGF-mediated ERK activation is also potentiated in HEK-293 cells by over-expression of PDE γ (Wan et al., 2001). They propose that GRK2 can potentiate ERK activation in response to EGF by a mechanism that involves GRK2 phosphorylation of PDE γ , which then promotes Src-mediated ERK activation (Wan et al., 2003) (section 1.1.7). I have performed immunofluorescence experiments in Hep2 cells to show that kinase dead GRK2 can potentiate ERK activation in response to EGF equally as well as wildtype GRK2 (Figure 5.6). I also find that kinase dead GRK2 co-immunoprecipitates with activated ERK in response to EGF treatment in HEK-293 cells (Figure 5.4). Therefore, GRK2 may be able to promote ERK activation in response to EGF via both kinase-dependent and kinase-independent mechanisms.

My data suggests that phosphorylation of GRK2 at Y13, Y86 and Y92 downstream of the EGF receptor might promote GRK2 binding to Rho_{GTP} (Figure 5.8). Given that the PDGF receptor also activates Rho (Schiller, 2006) and phosphorylates GRK2 at these sites, it is possible that recruitment of GRK2 promotes a scaffolding function of GRK2, as is the case downstream of the EGF receptor. However, in the case of the PDGF receptor this may serve only to offset the loss of Grb2/Sos1/Ras-mediated ERK activation caused by the reduced receptor activation, so there is no net affect on overall ERK activation. The PDGF receptor does, however, phosphorylate GRK2 at additional sites to those phosphorylated by the EGF receptor. This is evidenced by the fact that tyrosine phosphorylation of GRK2-Y/F is reduced by 80% relative to wildtype GRK2 in response to EGF (Chen et al., 2008), but only by 50% in response to PDGF (Wu et al., 2005). Phosphorylation of additional sites on GRK2 downstream of the PDGF receptor may result in a phosphorylation profile of GRK2 that does not facilitate Rho binding and ERK scaffolding. Further experiments will be required to ascertain which agonists can promote Rho-dependent ERK scaffolding by GRK2 (section 6.6). How signalling specificity may be achieved by GRK2 downstream of different agonists is discussed in the following section.

6.3. Signalling specificity downstream of GRK2.

Via activation of heterotrimeric G proteins and recruitment of GRKs and β -arrestins, GPCRs are capable of regulating many different signalling pathways, in response to agonist binding (DeWire et al., 2007; Reiter and Lefkowitz, 2006; Ribas et al., 2007). Several groups have proposed a phenomenon termed the ‘barcode

hypothesis' as a mechanism to explain how a GPCR can signal specifically to certain pathways in response to different ligands or in response to the same ligand but in a different cell type (Butcher et al., 2011; Nobles et al., 2011; Tobin et al., 2008). Briefly, the barcode hypothesis suggests that different agonists binding to a GPCR results in recruitment of different GRKs and thus a different phosphorylation profile (or barcode) on the GPCR. Subsequently, β -arrestins that are recruited to the phosphorylated GPCR will adopt different conformations depending on the phosphorylation barcode, resulting in different arrestin-dependent signalling outcomes downstream of the receptor. Similarly, in different cell types, which may contain different levels of each GRK, the same ligand-receptor binding event can result in a different phosphorylation barcode on the receptor and thus different signalling outcomes.

GRK2, like the arrestins, is also known to regulate multiple signalling pathways downstream of GPCRs and RTKs, for example by phosphorylating non-receptor substrates such as IRS-1 and ezrin (Penela et al., 2010a) (sections 1.1.5 - 1.1.7). In terms of MAPK signalling, I have shown that GRK2 can positively regulate ERK activation by acting as a Rho-dependent scaffold protein downstream of the EGF receptor, while in response to other stimuli, GRK2 has been shown to negatively regulate ERK activation, for example by sequestering MEK to inhibit chemokine-mediated ERK activation (Jurado-Pueyo et al., 2008; Kleibeuker et al., 2008). It is not fully understood how signalling specificity downstream of GRK2 can occur such that it can direct different signalling outcomes in response to different agonists or potentially in response to the same agonist but in different cell types.

In this thesis I have found that ERK MAPK scaffolding by GRK2 is dependent on Rho_{GTP} binding to the kinase (Figure 5.3). Thus, this particular function of GRK2 may be specific to receptors such as the EGF receptor that also activate Rho, while downstream of receptors that do not activate Rho, GRK2 may regulate ERK signalling via receptor desensitisation or other mechanisms previously described (section 1.1.7). It may also be possible for GRK2 to act as an ERK scaffold when recruited to a given receptor if the cell had previously received another signal to activate Rho, even if the receptor in question could not itself activate Rho. This would be an example of achieving signalling specificity by ‘cross-talk’. GRK2 does, however, negatively regulate ANG_{II}-induced ERK activation by phosphorylating and internalising the receptor in HEK-293 cells (Kim et al., 2005) and *in vivo* (Eckhart et al., 2002), despite the fact that the angiotensin receptor is also known to activate Rho (Bhattacharya et al., 2004). There may, therefore, be an added degree of specificity.

I have found that Rho_{GTP} binding to GRK2 is increased by stimulation of the EGF receptor and this is prevented by mutation of tyrosine residues 13, 86 and 92 within the GRK2 amino-terminal domain (Figure 5.8). This suggests that tyrosine phosphorylation of GRK2 is required for EGF-induced Rho_{GTP} binding and subsequent ERK scaffolding by GRK2. Therefore ERK scaffolding by GRK2 may be specific to receptors that both activate Rho and stimulate phosphorylation of GRK2 at these sites. GRK2 may not be tyrosine phosphorylated at residues 13, 86 and 92 in response to ANG_{II}, providing an explanation of why Rho_{GTP} binding and subsequent ERK scaffolding by GRK2 apparently does not occur downstream of the AT1R. It would be interesting to test whether this specificity for ERK scaffolding downstream of the EGF receptor versus the AT1R is lost if the three tyrosine

residues are mutated to aspartates (GRK2-Y/D). If this mutant mimics tyrosine phosphorylation at these sites, as has been reported (Penela et al., 2008) but not shown, it might enable binding to Rho_{GTP} in the absence of a signal to phosphorylate tyrosine residues 13, 86 and 92.

GRK2 is also phosphorylated by PKC at serine 29 in response to ANG_{II} (Malhotra et al., 2010). Phosphorylation at this site is known to increase GRK2 kinase activity (Krasel et al., 2001; Pronin et al., 1997) but it might also provide a potential mechanism by which ERK scaffolding may be prevented downstream of the angiotensin receptor. Maybe S29 phosphorylation downstream of ANG_{II} but not EGF prevents Rho binding to the GRK2 amino-terminal region. Again, further experiments with S29A and S29D mutants of GRK2 could be performed to test this hypothesis. Given that Src is also activated downstream of the AT1R (Kyaw et al., 2004) and GRK2 tyrosine residues 13, 86 and 92 are known to be phosphorylated by Src (Penela et al., 2001), S29 phosphorylation could prove to be the important factor in determining GRK2 signalling specificity (section 6.6).

6.4. GRK2 and hypertension.

I have found that VSMC thymidine incorporation downstream of the EGF receptor is Rho- and ERK-dependent and inhibited by siRNA knockdown of GRK2 (Figure 5.7), suggesting that Rho-dependent ERK scaffolding by GRK2 is required for proliferation of VSMCs. This might in part explain how mice with GRK2 over-expressed specifically in their VSMCs develop hypertension associated with vascular thickening (section 1.3.2) (Eckhart et al., 2002). The increased levels of GRK2 in these mice may result in increased ERK scaffolding and therefore

aberrantly high VSMC proliferation, manifesting in thickening of the VSM (Figure 6.1B). ERK scaffolding by GRK2 requires GRK2 binding to Rho_{GTP} (Figure 5.3), which is also implicated in hypertension (section 1.4). Mutation of tyrosine residues 13, 86 and 92 within the amino-terminal region of GRK2 abrogates EGF-stimulated Rho_{GTP} binding to GRK2 (Figure 5.8), suggesting that Rho_{GTP} binding is promoted by phosphorylation of GRK2 at Y13, 86 and 92. Therefore, if GRK2-mediated ERK scaffolding is important in hypertension, I would predict that VSMC-specific over-expression of a mutant of GRK2 that cannot bind to Rho or cannot be tyrosine phosphorylated may not cause aberrant VSMC proliferation and hypertension in mice. In the long term, inhibition of Rho binding to GRK2 might be a useful therapeutic approach for treating hypertension. This is clearly very speculative and experiments that could support a role for Rho-activated scaffolding by GRK2 in hypertension will be outlined in section 6.6.

Interestingly, while VSMC-specific over-expression of GRK5 in mice does increase blood pressure, it does not result in vascular thickening (Keys et al., 2005), suggesting that the effect on VSMC proliferation is specific to GRK2. I have found that MEK and ERK binding is specific to GRK2 and not GRK5 (Figure 4.7) despite the fact that GRK2 and GRK5 both bind to RhoA-V14 (Figure 3.3). The specificity of GRK2, and not GRK5, for promoting VSM thickening in mice could therefore be explained by the fact that only GRK2 is capable of scaffolding ERK. A comparison of ERK activation in response to EGF in VSMCs cultured from mice over-expressing GRK2 and GRK5 and VSMCs from GRK2 and GRK5 heterozygous mice, which have 50% less GRK expression, would indicate whether the magnitude of EGF-induced ERK activation directly correlates with levels of GRK2, but not GRK5, expression.

6.5. Experimental weaknesses of this thesis.

- The evidence for ERK scaffolding by GRK2 is based predominantly on experiments performed in cell lines. I have shown that GRK2 is required for EGF-induced thymidine incorporation in primary VSMCs (Figure 5.7) but further experiments in this primary cell line are required to confirm a role for GRK2 as a Rho-activated ERK MAPK scaffold in this cellular setting (section 6.6).
- The evidence that GRK2 is an ERK scaffold downstream of the EGF receptor mostly comes from experiments where GRK2 is over-expressed. GRK2 levels could be reduced by RNAi in HEK-293 cells to test whether endogenous GRK2 also acts as an ERK scaffold in response to EGF. EGF-induced ERK activation could also be compared in wildtype versus GRK2 heterozygous (+/-) MEFs. Again, these experiments would ideally be performed in primary VSMCs isolated from wildtype, GRK2 heterozygous or GRK2 over-expressing mice to help support a role for ERK scaffolding by GRK2 *in vivo* (section 6.6).
- I would predict that, as a scaffold protein, GRK2 can bind to Raf1, MEK1 and ERK2 simultaneously. Although GRK2 does co-immunoprecipitate with Raf1, MEK1 and ERK2 individually in the presence of RhoA-V14 (Figure 4.2D, E and F), I haven't proven that GRK2 can interact with all three simultaneously, or indeed that any of these components bind to GRK2 directly.
- The RhoABC inhibitor C3 is used throughout this thesis to demonstrate that the scaffolding role of GRK2 is Rho-dependent, however, C3 treatment does result in morphological changes to cells and it would be useful to inhibit GRK2 via

alternative potentially less intrusive techniques, such as RNAi of RhoA, RhoB and RhoC.

- Preliminary data suggests a role for tyrosine phosphorylation of GRK2 in promoting Rho_{GTP} binding and subsequent ERK scaffolding by GRK2. The current evidence for this is that EGF stimulation of cells promotes GRK2 binding to RhoA-V14 and P-ERK but fails to promote GRK2-Y13, 86, 92/F binding to RhoA-V14 and P-ERK. Further characterisation of the role of tyrosine phosphorylation in regulating the scaffolding function of GRK2 is required, as detailed in the following section.

6.6. Future directions.

Tyrosine phosphorylation and signalling specificity of GRK2

How GRK2 can act as an ERK scaffold specifically downstream of the EGF receptor while negatively regulating ERK downstream of other receptors, such as chemokine receptors (Jurado-Pueyo et al., 2008; Kleibeuker et al., 2008), requires further investigation. The effect of GRK2 on ERK activation downstream of a panel of different RTKs and GPCRs would enable us to test whether ERK scaffolding by GRK2 occurs only at receptors that activate Rho. The blots could also be probed with anti-phospho-tyrosine antibodies and antibodies raised against the PKA and PKC phosphorylation sites on GRK2 to test whether ERK activation by GRK2 downstream of different receptors correlates with a particular modification on GRK2. A definitive assessment of the residues modified on GRK2 downstream of the EGF receptor may require mass spectroscopy of GRK2 immunoprecipitated

from cells treated or not with EGF. Sites that are modified in response to EGF could then be mutated to test their importance in mediating ERK scaffolding by GRK2 downstream of the EGF receptor.

The preliminary data obtained using the GRK2-Y/F mutant (Figure 5.8) suggests that tyrosine phosphorylation of GRK2 downstream of the EGF receptor may be required to promote Rho_{GTP} binding and subsequent ERK scaffolding by GRK2. Given more time, the role of GRK2 tyrosine phosphorylation would be further examined. For example, I would predict that the GRK2-Y/F mutant that cannot be phosphorylated at the three amino-terminal tyrosine residues would fail to potentiate EGF-induced ERK activation when over-expressed in HEK-293 cells. It may also fail to translocate to the nucleus. Downstream of different agonists, modification of GRK2 at other sites such as the PKC site S29 may also affect Rho_{GTP} binding to GRK2. Different mutants of GRK2, including GRK2-Y/F and GRK2-Y/D as well as GRK2-S29A and GRK2-S29D, should be screened for their ability to interact with Rho, +/- EGF treatment. These mutants could also be screened for their ability to potentiate ERK activation downstream of EGF (and potentially other agonists) and for their ability to translocate to the nucleus in response to different agonists.

Assuming that amino-terminal tyrosine phosphorylation of GRK2 is required for full binding of GRK2 to Rho_{GTP}, this may cast some doubt on the data presented in figure 4.1. There I showed that GRK2 kinase activity and desensitisation of the angiotensin receptor is unaffected by over-expression of RhoA-V14 in cells. Although the role of the amino-terminal modification of GRK2 requires further characterisation, as discussed above, it is possible that GRK2 only binds weakly to RhoA-V14 in the absence of tyrosine phosphorylation. Thus, only a small

proportion of the GRK2 in the kinase assays and GPCR desensitisation experiments may have actually been bound to RhoA-V14. It is possible that RhoA-V14 binding would in fact affect GRK2 kinase activity and desensitisation of the AT1R if it could efficiently bind to GRK2. After all, one might imagine that GPCR phosphorylation by GRK2 would be compromised by Rho, Raf, MEK and ERK all binding within the vicinity of the GRK2 active site. It would be of interest to repeat the GRK2 kinase assays and AT1R desensitisation assays in the presence of EGF receptor over-expression and activation or using a GRK2-Y/D mutant, if this mutant is shown to mimic the effects of amino-terminal tyrosine phosphorylation of GRK2.

Rho-dependent functions of the other GRKs

GRKs 2, 3, 5, 6A, 6B and 6C all bind to RhoA-V14 (Figure 3.3) but GRK5 fails to bind to MEK and ERK in the presence of RhoA-V14 (Figure 4.7), suggesting that ERK scaffolding may be a specific function of GRK2, or of the GRK2 subfamily. Further experiments to test whether RhoA-V14 can bind to GRKs 1, 7 and 4 as well as efforts to understand the function of Rho binding to GRKs other than GRK2 are required. It would be interesting to test whether the catalytic domains of the other GRKs in isolation interact more strongly than with the full length GRK. This would indicate whether or not a conformational change is required for full Rho_{GTP} binding to the other GRKs, as is the case for GRK2. Modifications to the other GRKs, equivalent to the tyrosine phosphorylation of GRK2 amino-terminal residues, may promote Rho_{GTP} binding. Y92, one of the three PDGF/EGF-phosphorylated tyrosine residues from GRK2, is conserved in GRKs 1-6 (Lodowski et al., 2006). GRK5 is phosphorylated at this and other tyrosine

residues within its amino-terminal and catalytic domains downstream of the PDGF receptor (Cai et al., 2009). It would be interesting to test whether mutation of this residue affects Rho binding to the different GRKs. In terms of the function of Rho_{GTP} binding to the other GRKs, kinase assays could test the effect of Rho_{GTP} binding on their catalytic activity and further co-immunoprecipitation experiments could indicate whether Rho_{GTP} affects the interaction of other GRKs with any of their respective binding partners. For example, GRK5 is known to contain a functional nuclear localisation sequence (Johnson et al., 2004) and has been found to phosphorylate class II HDACs (Martini et al., 2008). Rho_{GTP} binding to GRK5 may affect its nuclear localisation and/or its ability to phosphorylate nuclear substrates. Alternatively, Rho_{GTP} binding to other GRK family members may promote an interaction with components of other MAPK cascades, such as ASK1, MKK4 and JNK of the JNK MAPK cascade or MKK3 and p38 of the p38 MAPK cascade.

Affect of GRK2 on the function of Rho

My work has focussed on the affects of Rho on the functions of GRK2. Equally, GRK2 binding to Rho_{GTP} may affect the kinetics of its inactivation, for example by acting as a GAP. *In vitro* Rho GTPase assays with purified RhoA in the presence or absence of purified GRK2 could be used to test whether GRK2 binding to RhoA affects its intrinsic GTPase activity. GRK2 binding may also affect the ability of Rho_{GTP} to interact with some of its known effectors, potentially by competing with other Rho effectors for binding to active Rho. For example, while I would predict that GRK2 siRNA would reduce EGF-induced ERK activation, EGF-induced cortical actin polymerisation, which is also RhoA-dependent (Malliri et al.,

1998), may increase in cells with reduced GRK2 levels due to a lack of competition for the Rho effectors involved. Given that, like DGK θ , GRK2 interacts with RhoA-V14 but not RhoA-V14/F25N (Figure 3.6), it likely interacts with Rho_{GTP} slightly differently than other effectors such as Rho kinase, which interact equally well with both mutants (McMullan et al., 2006). GRK2 may thus compete for binding to active Rho with some effectors and not others. Rho-dependent ERK activation by GRK2 downstream of the EGF receptor may therefore be predicted to be reduced by over-expression of DGK θ but not Rho kinase. Screening different Rho effectors for their ability to inhibit ERK scaffolding by GRK2 could help to classify Rho effectors as Rho kinase or DGK θ -like.

I have found that GRK2 interacts with the highly homologous RhoA, B and C, but not with the related but structurally divergent classical RhoGTPases Cdc42 or Rac (Figure 3.1). It would be interesting to test whether GRK2 can interact with RhoF and RhoD, two other classical Rho GTPases, which have roles in filopodium formation (Heasman and Ridley, 2008), or with the non-classical RhoGTPase RhoH, which has very low GTPase activity but is important in processes such as T cell receptor signalling (Dorn et al., 2007). If GRK2 is found to bind to any other Rho GTPase family members, some of these processes may also be affected by GRK2 over-expression and/or siRNA in cells.

The physiological significance of Rho-mediated ERK scaffolding by GRK2

Further characterisation of the potential *in vivo* roles of ERK scaffolding by GRK2 is required. In terms of a potential role in hypertension, VSMCs cultured from GRK2 heterozygous mice, which have 50% less GRK2 than wildtype cells,

and VSMCs from mice with VSMC-specific over-expression of GRK2 would be useful tools. A comparison of EGF-induced ERK activation and thymidine incorporation, in the presence/absence of Rho inhibitors, would help to support a role for Rho-activated ERK scaffolding by GRK2 in these primary cells. Given that GRK2 RNAi was found to reduce Rho- and ERK-dependent thymidine incorporation in VSMCs (Figure 5.7), GRK2 scaffolding may promote nuclear specific functions of ERK in these cells and GRK2 might be expected to translocate to the nucleus in response to EGF treatment, as is the case in HEK-293 cells (Figure 5.5). Nuclear translocation of GRK2 was not, however, observed in Hep2 cells (Figure 5.6), suggesting that this may be a cell line specific phenomenon. Immunofluorescence experiments should be performed in VSMCs to definitively assess whether or not EGF promotes GRK2 nuclear accumulation in these cells.

As has been discussed, I plan to characterise various mutants of GRK2, including GRK2-Y/F, GRK2-Y/D, GRK-S29A, GRK2-S29D, for their ability to interact with Rho and to promote EGF-mediated ERK activation. EGF stimulation of cells fails to promote binding of RhoA-V14 to GRK2-Y/F (Figure 5.8). It would therefore be interesting to test whether VSMCs over-expressing GRK2-Y/F respond differently to EGF as compared to VSMCs over-expressing wildtype GRK2, in terms of ERK activation or thymidine incorporation. If GRK2-Y/D interacts more strongly with Rho_{GTP} than the wildtype kinase, it may promote a greater increase in ERK activation in response to EGF than wildtype GRK2. Over-expression of kinase dead GRK2 would also be predicted to potentiate EGF-induced ERK activation in VSMCs, as it can do in Hep2 cells (Figure 5.6), however, it is not clear whether GRK2 kinase activity is required for nuclear localisation of GRK2 and/or ERK, or indeed whether nuclear localisation of GRK2 and/or ERK in this context has any

functional consequences. Maybe over-expression of kinase dead GRK2 would increase EGF-induced ERK activation in VSMCs to the same extent as wildtype GRK2 but without increasing thymidine incorporation, or promoting nuclear accumulation of GRK2 and/or ERK.

Ultimately, transgenic mice with VSMC-specific over-expression of GRK2-Y/F, or a mutant of GRK2 that is unable to bind to Rho, could be generated. It is known that VSMC-specific over-expression of wildtype GRK2 in mice results in hypertension, associated with thickening of the vascular wall and subsequent heart failure (Eckhart et al., 2002) (section 1.3.2). If Rho-activated ERK scaffolding by GRK2 is important in hypertension, I would predict that VSMC-specific over-expression of GRK2-Y/F, or a mutant of GRK2 that is unable to bind to Rho, in these mice would result in a reduced vascular thickening and lower blood pressure as compared to mice specifically over-expressing wildtype GRK2 in their VSMCs. If this is the case, the interaction between GRK2 and Rho may be a valid target for the treatment of high blood pressure.

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